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Date of mailing (day/month/year) 11 January 2001 (11.01.01)	
International application No. PCT/AU00/00497	**Applicant's or agent's file reference** 615375
International filing date (day/month/year) 24 May 2000 (24.05.00)	**Priority date** (day/month/year) 02 June 1999 (02.06.99)
Applicant HOLLOWAY, Barry, Charles	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

14 November 2000 (14.11.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer R. E. Stoffel
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Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing: 02 November 2000 (02.11.00)	
International application No.: PCT/AU00/00397	Applicant's or agent's file reference: 2269323/EJH
International filing date: 28 April 2000 (28.04.00)	Priority date: 28 April 1999 (28.04.99)
Applicant: ALTIN, Joseph et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International preliminary Examining Authority on:

10 October 2000 (10.10.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

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Telephone No.: (41-22) 338.83.38

PCT

INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 39/295, 39/39, C07K 17/06, 17/14, C12N 11/06, 11/14, G01N 33/483, 33/68

A1

(11) International Publication Number: WO 00/64471

(43) International Publication Date: 2 November 2000 (02.11.00)

(21) International Application Number: PCT/AU00/00397

(22) International Filing Date: 28 April 2000 (28.04.00)

(30) Priority Data:
PQ 0023 28 April 1999 (28.04.99) AU

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(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
With amended claims.

(54) Title: MODEL MEMBRANE SYSTEMS

(57) Abstract

The present invention relates generally to anchoring molecules to model and biological membrane systems, and to the use of anchored molecules in assays of inter molecular interactions and to modify biological responses. In one form, the present invention provides the basis of a novel method for the screening of drugs and other agents which affect intermolecular interactions. In another form, the invention provides a means of modifying the properties of biological and/or synthetic membranes and liposomes for the purpose of altering immunity when used as vaccines, or for the targeting of drugs and other agents to specific cells or tissues when administered *in vivo* for either therapeutic purposes or for modifying physiological responses or function.

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MODEL MEMBRANE SYSTEMS

FIELD OF THE INVENTION

The present invention relates generally to anchoring molecules to model and biological membrane systems, and to the use of anchored molecules in assays of inter molecular interactions and to modify biological responses. In one form, the present invention provides the basis of a novel method for the screening of drugs and other agents which affect intermolecular interactions. In another form, the invention provides a means of modifying the properties of biological and/or synthetic membranes and liposomes for the purpose of altering immunity when used as vaccines, or for the targeting of drugs and other agents to specific cells or tissues when administered *in vivo* for either therapeutic purposes or for modifying physiological responses or function.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

In biological systems such as cells, bacteria or viruses, surface biomolecules or receptors often exist as molecular structures consisting of two or more molecular components called subunits; these subunits may be identical, or molecularly distinct. The binding of natural ligand molecule(s) to receptor subunits may induce non-covalent aggregation or oligomerization of these receptor components. The oligomerization event is often an essential part of the mechanism by which the receptor can transduce transmembrane signals for triggering the induction of biological responses by the ligand molecule(s). In addition, the ability of certain receptors, or components thereof, to aggregate spontaneously may affect their ability to interact with ligands. Ligand molecules may be growth factors, cytokines, hormones, proteins, glycoproteins, polysaccharides, or any surface exposed or sub-cellular component of a cell, viral or subviral particle, or other infectious agent, which can bind to the receptor.

By virtue of their ability to undergo multimeric interactions, oligomerized receptors often have the potential to interact stably with ligands of low binding affinity. However, for many receptors the affinity of self-association or interaction with ligands is not high enough to allow detection using conventional binding techniques, which often require covalent labelling, solubilization with detergents, or immobilization of the receptor or ligand onto the solid sensing surface of an optical biosensor. These methods are suited to the study of relatively high affinity interactions and they generally rely on the ability of the molecules to interact in solution or to maintain stable interaction after cell disruption. Since the effective receptor/ligand concentration in solution is reduced compared to that on the two-dimensional surface of a cell (where molecules can oligomerize or cluster and interact stably with other molecules through multimeric interactions) these methods are limited in their ability to detect interactions of low affinity.

Atomic force microscopy (also known as scanning probe microscopy) allows three-dimensional imaging and measurement of structures ranging in size from atomic dimensions to microns, and is revolutionary in its ability to resolve structures never seen before (1). The development of optical biosensors has permitted the monitoring of the interaction between macromolecules in real time (2). To date, both of these techniques generally have been used with the receptor or the ligand molecule covalently attached to or immobilized onto a solid surface (1-4).

Recently a technique has been described in which the linkage of a recombinant hexa-histidine-tagged protein with nitrilotriacetic acid (NTA) is used to reversibly immobilize a hexa-histidine-tagged protein onto the solid sensing surface of a BIAcore surface plasmon resonance biosensor (5). The formation of a hybrid octadecanethiol/phospholipid membrane on the BIAcore sensing surface also has been described (6), enabling analysis of the binding of streptavidin to biotinylated phosphatidylethanolamine in the bilayer. In addition, the immobilization of histidine-tagged biomolecules to bilayer membranes *via* chelator lipids like NTA-dioctadecylamine has been demonstrated by epifluorescence microscopy and film balance techniques (7-8). These prior art techniques do not permit an analysis of an interaction of receptor domains under

conditions that mimic a cellular or sub-cellular membrane that allows lateral mobility of the receptors, and that allows for the possibility of studying receptor oligomerization and multimeric interactions with ligands. Furthermore, these prior art techniques do not permit screening for drugs or other agents that may influence such molecular interactions.

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There is a need, therefore, to develop new techniques to anchor molecules to membranous materials for use, for example, in devices for drug screening, in vaccine preparations, as therapeutic agents, as agents for modifying immunological responses and for targeting drug delivery systems.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

One aspect, the present invention provides a method of anchoring receptor domains onto a planar supported membrane comprising amphiphilic molecules arranged in a layer, wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group such that some of the metal chelating groups are oriented toward the outside surface of said membrane; which method comprises the step of interacting a receptor domain which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach to said membrane *via* the outwardly facing metal chelating residues of said membrane, such that the receptor domains are capable of lateral movement, dimerization or oligomerization, and interaction with a ligand molecule.

Another aspect, the present invention provides a method of anchoring receptor domains onto a lipid membrane such that the receptor domains are capable of lateral movement, said method comprising of:-

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of micelles (e.g. liposomes) from amphiphilic

molecules wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group, then interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a membrane layer to form in which some metal chelating residues attached to the amphiphilic molecules of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the metal chelating linkage to the outwardly-facing metal chelating residues of said membrane.

Another aspect the present invention contemplates a method of anchoring receptor domains onto a lipid membrane such that said receptor domains are capable of lateral movement to facilitate interactions with biomolecules in the membrane, said method comprising of:

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of micelles (e.g. liposomes) from a first phospholipid and a second phospholipid wherein the second phospholipid has been modified by a covalent attachment of a nitrilotriacetic acid (NTA), and then interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a phospholipid membrane layer to form in which some NTA residues attached to the second

phospholipid of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the NTA-metal chelating linkage to the outwardly-facing NTA residues of said membrane.

Yet another aspect of the present invention provides a method of assaying interactions between a receptor and a ligand, comprising the steps of:-

(A) anchoring a receptor to a lipid membrane by:-

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of micelles (e.g. liposomes) from amphiphilic molecules wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group, and then interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a membrane layer to form in which some metal chelating residues attached to the amphiphilic molecules of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the metal chelating linkage to the outwardly-facing metal chelating residues of said membrane;

(B) allowing said receptor molecules to interact and/or oligomerize on the membrane; and

(C) contacting said anchored receptor with an effective concentration of ligand for a time and under conditions sufficient for binding to occur and detecting said binding.

Still yet another aspect of the present invention contemplates a method of assaying interactions between a receptor and a ligand comprising the steps of:-

(A) anchoring a receptor to a lipid layer by:-

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of vesicles from a first phospholipid and a second phospholipid wherein said second phospholipid has been modified by covalent attachment of a metal chelating group such as nitrilotriacetic acid (NTA), and then interacting said micelle (e.g. liposome) suspension with an appropriate planar support such as glass or mica for a time and under conditions sufficient to allow a hydrocarbon phospholipid membrane layer to form on the support in

which some of the NTA residues attached to the second phospholipid of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the NTA-chelating linkage to the outwardly-facing NTA residues of said membrane;

(B) allowing said receptor molecules to interact and/or oligomerize on the membrane; and

(C) contacting said anchored receptor with an effective concentration of ligand for a time and under conditions sufficient for binding to occur and detecting said binding.

Even yet another aspect of the present invention there is provided a method of engrafting molecules onto liposomes said method comprising:

(i) preparing a suspension of liposomes with chelator lipid incorporated, and with or without an encapsulated drug or agent;

(ii) incubating the liposomes with a recombinant protein or target molecule bearing an appropriate metal affinity tag; and

(iii) if necessary, removing excess protein by washing, filtering or other washing means and suspending them in an appropriate solution.

Another aspect of the present invention, there is provided a method of "pasting" recombinant molecules directly onto biological membranes said method comprising:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or biological membranous structures with a suspension of the chelator lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the membranous structures with a solution of recombinant protein or target molecule possessing an appropriate metal affinity tag; and

(v) washing away excess or unbound soluble protein, and suspending the structures in a solution suitable for administration *in vivo*.

Still yet another aspect of the present invention contemplates a method for altering the immunogenicity of a target cell or membranous component thereof, said method comprising anchoring a molecule to the membrane of said target cell by:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or membranous structures with a suspension of the chelator lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the membranous structures with a solution of said molecule to be anchored; and

(v) washing away excess or unbound soluble molecule, and suspending the structures in a solution suitable for administration *in vivo*.

Even yet another aspect of the present invention contemplates a method of targeting a liposome or a membranous structure to a particular cell or tissue, said method comprising

anchoring or engrafting a molecule having a binding partner on the particular cell or tissue to be targeted by:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid; or

(ii) incubating a suspension of cells or membranous structures with a suspension of the chelator lipid to incorporate the lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the liposomes or membranous structures with a solution of molecules to be anchored; and

(v) washing away excess or unbound soluble molecule, and suspending the liposomes or structures in a solution suitable for administration *in vivo*.

Another aspect of the present invention contemplates a method of treatment, said method comprising administering to a subject an effective amount of a liposome preparation or membranous material comprising an active material and optionally an anchored or engrafted molecule having a binding partner or target tissue.

A further aspect of the present invention provides a vaccine composition comprising cells, liposomes, vesicles or membranous material having engrafted thereto molecules capable of modifying an immunological response to a subject to which the vaccine is administered, said vaccine further comprising one or more pharmaceutical carriers and/or diluents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram showing anchoring of a hexa-histidine-tagged protein to a model membrane formed on the glass sensing surface of an IAsys biosensor cuvette. As shown in Figure 1, to anchor hexa-histidine-tagged proteins onto a membrane for studying protein interactions with the IAsys biosensor, a lipid layer is first formed on the sensing surface of the biosensor cuvette using a known method which involves reacting the surface with a suspension of micelle (e.g. liposomes) made from 90% PC and 10% PE-NTA. The PE-NTA for these experiments was produced by first reacting PE with the bifunctional cross-linker bis(sulfosuccinimidyl)suberate, and then reacting the mixture with NTA; the NTA-PE product was purified by thin layer chromatography (and its identity confirmed by mass spectroscopy). The layer is then washed to remove excess micelle (e.g. liposome) suspension and equilibrated in Tris-buffered saline (TBS). In accordance with the present invention, the inventors show that this membrane can be used to anchor hexa-histidine-tagged molecules in a way that allows them to diffuse laterally and interact, thereby mimicking the cell surface.

Figures 2(a) and 2(b) are traces showing the change in refractive index as a function of time due to binding of molecules as monitored with an IAsys biosensor.

Figure 3 is a biosensor trace showing changes in refractive index due to additions made to an IAsys biosensor cuvette after reacting the sensing surface of the cuvette with a suspension of micelles (e.g. liposomes) to form a layer.

Figure 4 is an illustration of the way hexa-histidine-tagged molecules can be engrafted onto the membranes of cells or other membranous structures. The illustration depicts how recombinant receptors bearing a hexa-histidine tag can be engrafted onto biological membranes such as the plasma membrane of cells or the membrane of sub-cellular membranous structures and onto the surface of artificial vesicles or liposomes. The recombinant protein is engrafted onto the membrane structure through the binding of the hexa-histidine tag on the protein to the NTA metal chelating headgroup on the chelator lipid

(denoted NTA-DTDA; this may also be referred to as di-C14-NTA) which has been incorporated into the phospholipid membrane.

Figure 5 is the fluorescence profile, as measured by fluorescence-activated cell sorting, of P815 cells engrafted with biotinylated and hexa-histidine-tagged CD40 and B7.1 molecules and then stained with streptavidin-FITC.

Figure 6 is a graphical representation showing an induction of tumor-specific cytoxicity in T lymphocytes isolated from mice vaccinated with tumor cells bearing engrafted co-stimulator molecules. Syngeneic DBA/2 mice were immunized subcutaneously with either PBS or 1 x 10^5 γ-irradiated P815 cells engrafted with the recombinant proteins: EPOR-6H, B7.1-6H, and B7.1-6H plus CD40-6H, as indicated. Spleens were removed from the mice 14 days after immunization, and T lymphocytes (effector T cells) were isolated, suspended in incubation medium and aliquoted into 24-well flat-bottom plates at a concentration of 1 x 10^5 cells/well, and then co-cultured with 1 x 10^5 γ-irradiated native P815 cells. After 5 days co-culture at 37°C in the presence of 5% CO_2, the cells were incubated with ^{51}Cr-labelled native P815 cell targets for 6 hrs at 37°C at the indicated E:T ratio, before harvesting the supernatants and determining the amount of ^{51}Cr released through specific lysis. Results are expressed as the percentage specific lysis ± SEM, calculated as described in the Materials and Methods.

Figures 7(a) and (b) are graphical representations showing induction of tumor immunity by immunization with P815 tumor cells engrafted with recombinant co-stimulatory molecules. Mice were immunized by injection of either PBS or 1 x 10^5 γ-irradiated P815 cells engrafted with recombinant protein(s) including: EPOR-6H, B7.1-6H, and B7.1-6H plus CD40-6H, as indicated. Two weeks after injection the mice in each group were challenged with 1 x 10^5 native P815 cells by subcutaneous injection, and then monitored for tumor growth and survival. Each point in (A) represents the mean tumor diameter for each group of mice as a function of time for the first five weeks. The data in (B) show the percentage survival of the animals with time.

Figure 8 is a graphical representation showing that binding of fluorescently-labelled liposomes to D10 cells (murine CD4+ T cells) is significantly greater when the liposomes are engrafted with either of the co-stimulatory molecules CD40 and B7.1, than when engrafted with a control protein EPOR. (Note that the D10 cells express ligands for B7.1 and CD40 but no ligand for EPOR.) The liposomes, composed of the lipids: PC:NTA-DTDA:FITC-PE (10:1:0.1 molar ratio), were engrafted with one or more recombinant protein (as indicated, EPOR, B7.1 and CD40) each bearing a hexa-histidine tag. The fluorescence profile of the cells in each condition (which reflects the extent of binding of the liposomes to the cells) was determined by fluorescence-activated cell sorting; the background fluorescence of cells (indicated "cells") is shown for comparison. The results indicate that the binding of liposomes engrafted with an appropriate recombinant protein is specific for the type of engrafted protein and, therefore, that liposomes bearing engrafted recombinant proteins can be targeted to cells expressing the appropriate cognate receptor.

Figure 9 is a graphical representation showing that synthetic liposomes engrafted with the co-stimulatory molecules B7.1 and CD40 can specifically stimulate the adherence of D10 cells (murine CD4+ T cells) to the culture dish. Cultured D10 cells were suspended in complete growth medium (RPMI 1640 plus 10% v/v FCS, 50 U/ml IL-2, antibiotics and 50 μM β-mercaptoethanol). The recombinant proteins EPOR, CD40 and B7.1 (each with a hexa-histidine tag) were mixed with the cells either in soluble form (as indicated sEPOR, sB7.1 and sCD40) or engrafted onto liposomes composed of PC and NTA-DTDA (10:1) (as indicated NTA-DTDA-EPOR, NTA-DTDA-B7.1 and NTA-DTDA-CD40), before plating the cells into separate wells of a 12-well Linbro tissue culture plate and incubating in growth medium for 2 hrs at 37°C. After the incubation, the non-adherent cells were removed from the wells by washing three times with PBS, and the remaining adherent cells were removed with a solution of 1 mM EDTA and counted microscopically. The data shows the proportion of adherent cells for each condition. The results demonstrate that liposomes bearing engrafted co-stimulatory molecules (i.e. B7.1 and/or CD40) can be used to modify immunological responses.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In one embodiment, the present invention contemplates a method of anchoring the extramembranous or transmembrane domains of receptors to overcome one or more of the foregoing shortcomings of the prior art.

In another embodiment, the subject invention provides a method of assaying interactions between membrane anchored molecules and between anchored molecules and molecules capable of interacting therewith. More particularly, the present invention is useful to study interactions between extramembranous or transmembrane domains of receptors, and between receptor domains and a ligand(s), by anchoring the receptor domain(s) onto a fluid membrane system. Receptor domains may also be composed of proteins, glycoproteins or proteoglycans, oligosaccharides, or fragments or functional equivalents thereof.

An important application of the present invention, therefore, is in providing the technology for the screening of agents or drugs which affect either induced or spontaneous receptor aggregation and, hence, which influence receptor function. The present invention may also be used in the screening for drugs which disrupt pre-existing aggregates of receptor subunits, such as the normally associated α and β subunits of the MHC class II molecules described below.

The present invention is useful to monitor the spontaneous or ligand induced aggregation/ disaggregation processes of receptors or protein fragments which are not normally membrane associated, once they are engineered to have a hexa-histidine tag and anchored to the membrane as described below for the cell surface derived molecules CD4 and MHC class II. Alternatively, a receptor domain or related protein fragment can be engineered to contain a suitably located strand which spontaneously inserts into the outermost leaflet of the membrane layer. The application of this system includes drug screening and surveying for new ligand species or biochemical agents (e.g. enzymes) which induce modifications that affect aggregation processes.

Thus, the present invention is directed to a method of anchoring receptor domains (albeit extramembranous or transmembrane) onto a membrane such that said receptor domains are capable of lateral movement to facilitate interactions with biomolecules in the membrane.

Accordingly, the present invention provides a method of anchoring receptor domains onto a planar supported membrane comprising amphiphilic molecules arranged in a layer, wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group such that some of the metal chelating groups are oriented toward the outside surface of said membrane; which method comprises the step of interacting a receptor domain which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach to said membrane *via* the outwardly facing metal chelating residues of said membrane, such that the receptor domains are capable of lateral movement, dimerization or oligomerization, and interaction with a ligand molecule.

A preferred metal chelating group for use in the present invention is nitrilotriacetic acid (NTA).

The membrane may be formed from a suspension of micelles (e.g. liposomes) from the amphiphilic molecules, wherein a proportion of the amphiphilic molecules has been modified by covalent attachment of a metal chelating group. Alternatively, the membranes are formed by the addition of water or aqueous buffers to a solution of amphiphilic molecules in an organic solvent.

Another aspect of the invention provides a method of anchoring receptor domains onto a membrane such that the receptor domains are capable of lateral movement, said method comprising of:-

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in

an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of micelles (e.g. liposomes) from amphiphilic molecules wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group, then interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a membrane layer to form in which some metal chelating residues attached to the amphiphilic molecules of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the metal chelating linkage to the outwardly-facing metal chelating residues of said membrane.

The amphiphilic molecules are normally surfactant molecules having a hydrophilic "head" portion and one or more hydrophobic "tails". Surfactants may be any of the known types, i.e. cationic (e.g. quaternary ammonium salts), anionic (e.g. organosulfonate salts), zwitterionic (e.g. the phospholipids: phosphatidylcholines and phosphatidylethanolamines), membrane spanning lipid, or non-ionic (e.g. polyether materials).

The membrane may be comprised of more than one type of amphiphilic molecule. In a preferred embodiment, the membrane is comprised of a first phospholipid and a second phospholipid.

Thus, in a preferred form, the present invention contemplates a method of anchoring receptor domains onto a lipid membrane such that said receptor domains are capable of

lateral movement to facilitate interactions with biomolecules in the membrane, said method comprising of:

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of micelles (e.g. liposomes) from a first phospholipid and a second phospholipid wherein the second phospholipid has been modified by a covalent attachment of a nitrilotriacetic acid (NTA), and then interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a phospholipid membrane layer to form in which some NTA residues attached to the second phospholipid of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the NTA-metal chelating linkage to the outwardly-facing NTA residues of said membrane.

The method of this aspect of the present invention is particularly useful for anchoring extramembranous or transmembrane receptor domains.

In a further preferred form, the first phospholipid is phosphatidylcholine (PC) and the second lipid is phosphatidyl-ethanolamine-NTA (PE-NTA) and the ratio of PC:PE-NTA (w/v) is about 10:1. However, the first phospholipid can be any phospholipid or hydrocarbon capable

of forming a lipid layer; and the second phospholipid can be any lipid with a metal chelating headgroup which can be used to anchor receptor domains using a suitably engineered tag on the domain. In addition, the ratio of the first to the second phospholipid can be varied depending on the desired density of receptor domain molecules to be achieved on the membrane layer.

In one form of the present invention, the support is the glass-like sensing surface of an optical biosensor cuvette or a mica surface suitable for analysis by atomic force microscopy. Preferably, the support is a planar glass or mica surface, but could be any surface which can form a substrate for a supported planar lipid membrane layer (6, 9, 10).

In another form, the surface may be a layer of glass, gold or any material which can be reacted with a solution of a hydrocarbon-containing compound (e.g. octadecyltrichlorosilane and octadecanethiol), capable of attaching hydrocarbon chains to said surface (6, 11), such that chains of hydrocarbon are oriented away from the surface of said support to form a lipid monolayer or a hydrophobic surface which is capable of reacting with a suspension of phospholipid micelles (e.g. liposomes) to form a planar membrane layer (6, 9, 10). In these forms the NTA residues attached to the second phospholipid of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane. Such surfaces may be the sensing surface of optical biosensors that are capable of monitoring the interaction of receptor domains anchored on the membrane with ligand molecules in solution.

Preferably, the polypeptide tag comprises a sequence of at least six amino acid residues such as a hexa-histidine molecule, but can be any sequence of amino acids that can bind strongly through the formation of a complex with the metal chelating component of a lipid containing a metal chelating group such as NTA. In one application of the subject invention the molecule is a transcription factor molecule. In another form of the instant invention, the molecule is a receptor. More particularly, the receptor may be any cell surface receptor such as the human cell surface molecule CD4 or human MHC class II molecule, or domains of such receptors.

Accordingly, in another aspect, the present invention provides a method of assaying interactions between a receptor and a ligand, comprising the steps of:-

(A) anchoring a receptor to a membrane by:-

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of micelles (e.g. liposomes) from amphiphilic molecules wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group, and then interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a membrane layer to form in which some metal chelating residues attached to the amphiphilic molecules of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the metal chelating linkage to the outwardly-facing metal chelating residues of said membrane;

(B) allowing said receptor molecules to interact and/or oligomerize on the membrane; and

(C) contacting said anchored receptor with an effective concentration of ligand for a time and under conditions sufficient for binding to occur and detecting said binding.

In one embodiment, the present invention contemplates a method of assaying interactions between a receptor and a ligand comprising the steps of:-

(A) anchoring a receptor to a lipid layer by:-

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of vesicles from a first phospholipid and a second phospholipid wherein said second phospholipid has been modified by covalent attachment of a metal chelating group such as nitrilotriacetic acid (NTA), and then interacting said micelle (e.g. liposome) suspension with an appropriate planar support such as glass or mica for a time and under conditions sufficient to allow a hydrocarbon phospholipid membrane layer to form on the support in which some of the NTA residues attached to the second phospholipid of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane;

(ii) interacting the receptor domain to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the NTA-chelating linkage to the outwardly-facing NTA residues of said membrane;

(B) allowing said receptor molecules to interact and/or oligomerize on the membrane; and

(C) contacting said anchored receptor with an effective concentration of ligand for a time and under conditions sufficient for binding to occur and detecting said binding.

In the case where the solid support (e.g. gold) may not be suitable for layer formation directly from the vesicle suspension, steps (i) to (iii) above are preceded by a step to form a monolayer or hydrophobic surface on the solid support. This is achieved by reacting the solid support with a solution of a hydrocarbon-containing compound capable of attaching hydrocarbon chains to its surface, such that chains of hydrocarbon are oriented away from the surface of said support to form a lipid monolayer or a hydrophobic surface. The surface is then reacted with a suspension of phospholipid amphiphilic molecule micelles (e.g. liposomes), preferably micelles (e.g. liposomes) [as in steps (i)-(ii) above] for a time and under the conditions necessary to form a membrane layer. The metal chelating, preferably NTA residues attached to the amphiphilic molecules such as a second phospholipid will be oriented toward the outside surface of said membrane, and may be used to anchor molecules with a hexa-histidine tag [as described in (iii) above]. Such surfaces may be the sensing surface of optical biosensors that are capable of monitoring the interaction of receptor domains anchored on the membrane with ligand molecules in solution.

Preferably, the oligomerization or self-association of the receptor and binding to the ligand is detected by atomic force microscopy or by an optical biosensor technique.

The present invention thus provides for the anchoring of receptor domains, proteins, glycoproteins and polysaccharides, onto lipid layers formed on mica, glass, gold or any other appropriate surface, that enable the molecules to diffuse laterally and interact. This technology is ideal in a preferred embodiment for studying the interaction between receptor domains and between receptor domains and ligands in a membrane system using optical biosensor and atomic force microscope techniques.

Receptor domains can be engineered to have a hexa-histidine COOH-tail, or NH2-tail using standard recombinant DNA techniques. A hexa-histidine tag also may be covalently attached to polysaccharides and other molecules by chemical means.

Optical biosensors used in connection with this invention measure the change in refractive index due to the binding of a soluble macromolecule with a molecule immobilized onto the solid sensing surface and can be used to measure the binding affinity of the interacting molecules. The IAsys biosensor employs a sample cuvette which has a resonance mirror as the sensor surface. The glass-like sensing surface of the IAsys biosensor is ideal for reacting with a suspension of micelles (e.g. liposomes) to form a membrane layer on the sensing surface.

The present invention may also be used with a BIAcore surface plasmon resonance biosensor which utilizes a gold sensing surface. The gold is first reacted with octadecanethiol to form an octadecane monolayer, and then reacted with a suspension of micelles (e.g. liposomes) to form the membrane layer.

Atomic force microscopes can be used for mapping molecular structures on the model membrane layers containing a limited number of molecular species. Such layers can be formed either directly on mica or glass surfaces by incubation with a suspension of micelles (e.g. liposomes). A hydrophobic monolayer also can be formed on glass or gold surface by treating with an appropriate reagent, and a hybrid membrane layer formed by reacting the surface of the monolayer with a suspension of micelles (e.g. liposomes) composed of the amphiphilic molecules, preferably an appropriate mixture of PC and PE-NTA to form the layer.

The anchoring of hexa-histidine tagged receptors onto model membrane systems of the present invention is also useful to anchor or engraft receptors and other molecules onto liposomes or vesicles which through the specificity of the engrafted molecules can target and deliver drugs, DNA/RNA or any therapeutic agent that can be encapsulated into the liposomes, to specific cell types or tissues when the liposomes are administered *in vivo*.

Recombinant molecules also can be engrafted onto synthetic liposomes or vesicles for the purpose of developing vaccines to produce specific biological or therapeutic effects.

According to this aspect of the present invention, there is provided a method of engrafting molecules onto liposomes said method comprising:

(i) preparing a suspension of liposomes with chelator lipid incorporated, and with or without an encapsulated drug or agent;

(ii) incubating the liposomes with a recombinant protein or target molecule bearing an appropriate metal affinity tag; and

(iii) if necessary, removing excess protein by washing, filtering or other washing means and suspending them in an appropriate solution.

In a preferred embodiment, the molecules may be anchored or engrafted onto liposomes by the following method:-

(i) preparing a suspension of liposomes from an appropriate mixture of a chelator lipid such as di-tetradecylamine nitrilotriacetic acid (NTA-DTDA) and 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) at a concentration of ~ 0.5 mM in aqueous solution such as PBS (phosphate buffered saline) containing a concentration of Ni2+ or Zn2+ approximately equal to that of the NTA-DTDA. The liposomes can be produced by sonicating the mixture for 5-10 mins at a temperature above the Tm. Alternatively, the liposomes can also be produced by dissolving the lipids in an ethanolic solution and then dispersing in aqueous buffer, or by extruding an acqeous suspension of the lipids through polycarbonate or similar filter of suitable pore size. Typically, the ratio of NTA-DTDA:POPC can be 5:1, but can be different;

(ii) washing the liposomes by pelleting (by centrifuging at ~95,000 x g for 30 min at 4°C) and removing the supernatant, or by filtration techniques, and then suspending

the liposomes in an appropriate volume of the buffering solution to facilitate incubation with the tagged protein(s);

(iii) incubating the liposomes with a recombinant protein (e.g. human hexa-histidine-tagged VEGF, vascular endothelial growth factor), or a combination of different recombinant proteins, each bearing a hexa-histidine or other suitable metal affinity tag to allow it to be anchored onto the liposomes; and

(iv) removing excess soluble or unincorporated protein by washing the liposomes as in step (ii) above, then suspending them in PBS or other buffer solution suitable for administration *in vivo*.

Other combinations and different lipids can also be used in conjunction with the chelator lipid to give the liposomes specific properties. For example, the ganglioside GM1 or derivatives polyethylene glycol can be included in the mixture (in step (i) above) to produce liposomes with "stealth" properties (12) to avoid them being taken up by macrophages or by the liver or spleen when used as vaccines *in vivo*. Also, step (i) can be carried out in the presence of a drug, DNA or other therapeutic agent for the purpose of encapsulating the material and permitting it, when administered *in vivo*, to be delivered to specific cells or tissues defined by the specificity of the engrafted molecule(s). For example, liposomes with engrafted VEGF (vascular endothelial growth factor) can be used to target angiogenic epithelium which is known to express the VEGF receptor and is required for tumor growth. Liposomes with engrafted VEGF, therefore, can be used to deliver a cytotoxic drug or agent that can block the growth of new blood vessels needed for the growth of tumors. The cytotoxic drug or agent is encapsulated within the liposome.

Current methods of modifying the surfaces of cells to be used as vaccines for altering immunity to disease (e.g. the immune response to tumors - see below) generally require the transfection or genetic manipulation of the tumor cells, to induce them to express one or more specific protein(s) on their surface (13-15). For example, in both animal and human tumor models evidence suggests that the transfection of tumor cells with genes inducing

them to express T cell costimulator molecules like B7-1 (CD80), B7-2 (CD86), CD40 and ICAM-1 on their surface, may be a useful approach to prepare the cells for use in vaccinations to enhance tumor immunity in the tumor bearing host (16-22). Unfortunately, in a clinical setting, such as in the treatment of cancer in humans, the transfection of tumor cells with such genes can be time consuming and inconvenient. Thus, the frequency of transfection is generally low, and successful transfection with multiple genes (to induce expression of multiple proteins on the tumor cell surface) can be difficult to achieve. Furthermore, transfection techniques, even when carried out by the use of seemingly harmless viral vectors, can be associated with risks to the patient owing to the difficulty in precisely controlling the expression of the gene or its integration into the genome.

The present invention further provides a more convenient and safe method of engrafting co-stimulatory and other molecules directly onto the surfaces of cells (such as tumor cells) and other membranous structures (either biological or synthetic), that can be used as vaccines to enhance or modify immunity to tumors and other diseases in humans.

The method of anchoring receptors onto model membrane systems to assay for inter molecular interactions and drug screening can be used to anchor or "engraft" molecules directly onto biological membranes (e.g. the membranes of cells or subcellular particles), once a chelator lipid (e.g. NTA-DTDA) has been incorporated into the membranes, thereby providing a convenient way of engrafting recombinant molecules possessing a hexa-histidine or other suitable metal affinity tag, directly onto the membrane surface.

In this aspect of the present invention, there is provided a method of anchoring or engrafting recombinant molecules directly onto biological membranes said method comprising:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or biological membranous structures with a suspension of the chelator lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the membranous structures with a solution of recombinant protein or target molecule possessing an appropriate metal affinity tag; and

(v) washing away excess or unbound soluble protein, and suspending the structures in a solution suitable for administration *in vivo*.

In a preferred embodiment, the invention allows the engrafting of molecules onto cells and other membranous structures using the following method:-

(i) washing a suspension of the cells or membranous structures with PBS or other aqueous buffer solution to remove excess soluble and/or loosely bound proteins. This can be carried out by pelleting the structures by appropriate centrifugation (e.g. 5 min at 200-500 x g for murine and human cells), and then resuspending them in PBS; depending on the structures, excess soluble or loosely bound proteins may be removed by filtering or other washing means;

(ii) preparing a suspension of chelator lipid (e.g. NTA-DTDA, at a concentration of ~0.1 mM) in PBS containing an approximately equal concentration of either Zn2+ or Ni2+ by sonicating for 5-10 min an appropriate quantity of the lipid in the PBS solution. Other lipids or phospholipids (e.g. POPC) or other agents also can be included with the chelator lipid to promote the fusion and incorporation of the liposomes into the membrane structures;

(iii) incubating the cells or membranous structures with a suspension of the chelator lipid (e.g. 0.1 mM NTA-DTDA) in PBS for a suitable period of time and temperature (e.g. 30 min, at 37°C) to allow some of the lipid in the suspension to fuse and/or become incorporated into the structures. Note: the incubation conditions employed can be altered to suit the nature of the chelator lipid used and the particular membrane structure into which the lipid is to be incorporated; also, incubations or wash steps in

buffer containing additives such as polyethylene glycol can be used to promote lipid fusion and incorporation;

(iv) removing unincorporated lipid from the mixture by washing the membranous structures with PBS by pelleting and washing as in step (i) above;

(v) incubating the washed structures containing incorporated chelator lipid with a solution of a recombinant protein, or a solution of a mixture of different recombinant proteins, each containing a hexa-histidine or any other appropriate metal affinity tag; and

(vi) washing the cells or structures with PBS (as in step (i) above) to remove excess or unbound soluble recombinant protein.

A similar procedure can be used to engraft tagged proteins to any cells, or subcellular membranous components. The structures so treated will contain a modified surface due to the engrafted protein, and can be used in vaccinations to alter immunological responses *in vivo*. The structures when administered *in vivo* also can be used to target a particular cell type or tissue within the body thereby altering the function of these cells. For example, the engrafting of tumor cells with molecules known to bind receptors on dendritic cells can be employed to direct the engrafted tumor cells to the dendritic cells to enhance tumor antigen presentation and hence immunological responses against the tumor.

In this form, the present invention contemplates a method of anchoring or engrafting recombinant receptors and other molecules directly onto biological membranes, such as the membrane of cells, and, hence, of modifying the properties of such membranes. In particular, the instant invention provides the basis of a convenient strategy for modifying the surfaces of cells (e.g. tumor cells), any cellular or subcellular membranous component, infectious agent or particle (e.g. bacteria), as well as any biological or synthetic membrane including synthetic vesicles or liposomes, into which the chelator lipid can be incorporated. In all these instances, the recombinant protein is engrafted by the formation of a metal chelating linkage

between a peptide tag on the protein, and the NTA headgroup on the chelator lipid incorporated into the membranous structure. The biological membrane being modified by the anchoring of any recombinant protein, glycoprotein and any other molecular structure possessing an appropriate tag, and designed to enhance immunity to diseases when used either as a vaccine, or as an agent to target delivery of these biological membranes to specific cells and tissues when administered *in vivo*.

Examples of suitable molecules in accordance with this aspect of the present invention includes therapeutic molecules, pharmaceutical compounds and nucleic acid molecules such as RNA and DNA. A particularly useful molecule is VEGF or its homologue. VEGF and its homologues are also useful for targeting liposomes to cells carrying VEGF receptors. Accordingly, the molecules contemplated by this aspect of the present invention include molecules having binding partners on target tissue. Preferably, the molecules are engrafted, anchored or encapsulated within the liposome.

A further aspect of the present invention provides for altering the immunogenicity of a target cell. This may be readily accomplished by introducing foreign polypeptides, polysaccharides, glycoproteins, receptors, ligands and other molecules. Altering the immunogenicity of cells such as tumor cells is a useful way of enhancing an immune response against tumor cells.

Accordingly, another aspect of the present invention contemplates a method for altering the immunogenicity of a target cell or membranous component thereof, said method comprising anchoring a molecule to the membrane of said target cell by:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or membranous structures with a suspension of the chelator lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the membranous structures with a solution of said molecule to be anchored; and

(v) washing away excess or unbound soluble molecule, and suspending the structures in a solution suitable for administration *in vivo*.

In a more particular embodiment, the present invention contemplates a method of targeting liposomes or membranous structures to a particular cell type or tissue, said method comprising anchoring or engrafting a molecule having a binding partner on the particular cells or tissue to be targeted by:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or membranous structures with a suspension of the chelator lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the liposomes or membranous structures with a solution of molecules to be anchored; and

(v) washing away excess or unbound soluble molecule, and suspending the structures in a solution suitable for administration *in vivo*.

As stated above, the methods of the present invention provides methods for altering the immunogenicity of cells. Accordingly, the present invention provides a method of treatment, said method comprising administering to a subject an effective amount of a liposome preparation or membranous material comprising an active material and optionally an anchored or engrafted molecule having a binding partner or target tissue.

Examples of active material include but are not limited to a recombinant polypeptide, co-

stimulatory molecule, therapeutic drug or nucleic acid molecule. In one example, VEGF is incorporated into a liposome to target a cytotoxic drug to block the growth of new blood vessels needed for the growth of tumors.

Another aspect of the present invention provides a vaccine composition comprising cells or membranous material having engrafted thereto molecules capable of modifying an immunological response to a subject to which the vaccine is administered, said vaccine further comprising one or more pharmaceutical carriers and/or diluents. Preferably, the molecules engrafted to the cells or membranous material are co-stimulatory molecules. Furthermore, the vaccine is preferably produced by the steps comprising:-

(i) incubating the cells or membranous material with a chelator lipid such as NTA-DTDA to allow the lipid to incorporate in the cells or membranes;

(ii) washing off any unincorporated lipid by centrifugation or filtration and resuspension of the structures in the appropriate solution or buffer;

(iii) incubating the membranous structures with incorporated chelator lipid with said molecules to be engrafted; and

(iv) washing off unincorporated molecular material.

Accordingly, the present invention enables the incorporation of chelator lipids like NTA-DTDA into tumor cell membranes, followed by the engraftment of recombinant co-stimulatory and/or other molecules (or combinations of molecules) with an appropriate tag, may be a convenient approach in the development of cell-based vaccines to enhance tumor immunity. Analogous to its demonstrated ability to alter tumor immunity therefore, the technique also can be expected to provide a convenient approach to engraft specific co-stimulatory and/or other cell surface molecules (or combinations of such molecules) onto other cell types including T cells, B-cells and dendritic cells, to see what role such molecules might play in regulating immune function. In addition to its potential use in cancer

immunotherapy, therefore, the technique described herein will have application to areas that could significantly enhance our understanding of immune function.

The present invention further provides a use of membrane system comprising an agent engrafted, encapsulated and/or anchored thereto in the manufacture of a medicament for modifying an immune response in an animal.

Preferred animals in accordance with the present invention are humans, primates, livestock animals, laboratory test animals and captured wild animals.

Terms such as "anchoring" and "engrafting" may be used interchangedly throughout the subject specification. The term "engrafting" also encompasses the term "grafting". The terms "membrane" and "lipid membrane" include reference to biological and synthetic membranes as well as lipid layers. The term "layer" or its plural "layers" includes monolayers and multilayers such as bilayers. A "chelator lipid" may be any suitable chelating lipid such as but not limited to NTA-DTDA.

The materials and methods below relate to some of the Examples which follow.

Reagents

Analytical grade reagents were used in all experiments. Paraformaldehyde was obtained from BDH Chemicals. $ZnSO_4$ was used for all additions of Zn^{2+} to buffers and growth media. RPMI 1640 and EMEM (Eagles minimal essential medium) both were obtained from Gibco (Life Technologies, Melbourne, Australia). Fetal calf serum (FCS) was obtained from Trace Scientific (Noble Park, Vic. Australia). Sulfo-NHS-LC-Biotin was obtained from Pierce (Rockford, IL). $Na^{51}CrO_4$, [^{3}H]-thymidine, and fluorescein isothiocyanate (FITC)-conjugated streptavidin (streptavidin-FITC) were obtained from Amersham (UK). Dioleoyl-phosphatidylethanolamine (DOPE), α-palmitoyl-β-oleoyl-phosphatidylcholine (POPC), dimyristoyl-phosphatidylcholine (DMPC), Isopaque, ficoll, propyl gallate, and the polyethylene glycol (PEG) preparations PEG_{400}, PEG_{600}, PEG_{900}, and PEG_{1500}, were all obtained from Sigma-Aldrich Pty Ltd (Castle Hill, NSW, Australia). MicroScint scintillation fluid and other items such as filters and seals for 96-well plates for use with the Topcount NXT microplate scintillation counter were obtained from Canberra Packard (Canberra, ACT, Australia).

Mice and Cell lines

Female or male DBA/2J mice (H-2^d), were used for isolation of lymphoid tissue (spleen) for T cell proliferation, measurement of T cell cytotoxicity, and for vaccination and monitoring of tumor growth *in vivo*. C57BL/6J mice (H-2b) were used in experiments assessing allogeneic stimulation of T cell proliferation. The mice were used at 6-8 weeks of age and were obtained from the Animal Breeding Establishment, John Curtin School of Medical Research (JCSMR), Australian National University (ANU), Canberra. The murine cell lines, P815 [murine DBA/2 (H-2^d) mastocytoma] and EL4 [murine C57BL/6 (H-2^b)] T cell lymphoma, were obtained, respectively, from Drs P. Waring (Division of Immunology and Cell Biology, JCSMR) and H. O'Neill (Division of Biochemistry and Molecular Biology), ANU. Both cell lines were cultured in complete medium consisting of EMEM containing

10% v/v FCS.

Synthesis of NTA-DTDA

The chelator-lipid nitrilotriacetic acid didtetradecylamine (NTA-DTDA), consisting of a nitrilotriacetic acid (NTA) head-group covalently linked to ditetradecylamine (DTDA) was synthesized by Dr C. Easton (Research School of Chemistry, ANU) following a procedure similar to that previously described (25). Briefly, the DTDA was synthesized from bromotetradecane and ammonia. DTDA was then *N*-succinylated with succinic anhydride to produce *N*-succinyl-DTDA (DTDA-suc), which was reacted with *N*-hydroxysuccinimide (NHS) to produce *N*-[(hydroxysuccinimidyl)succinyl]-DTDA (DTDA-suc-NHS). The succinimidyl group of DTDA-suc-NHS was replaced with a N^{α}-tert-butyloxycarbonyl-lysine (N-Boc-lys) group, and the butyloxycarbonyl (Boc) group was removed to produce N^{ε}-[(DTDA) succinyl]-L-lysine (DTDA-suc-Lys). DTDA-suc-Lys was finally reacted with bromo-acetic acid to produce N^{α},N^{α}-bis[carboxymethyl]-N^{ε}-[(DTDA)suc]-L-lysine, which will be referred to as NTA-DTDA. The purity of each product was measured by thin layer chromatography, and the identity of the final product was confirmed by nuclear magnetic resonance spectroscopy, Fourier transformed infrared spectroscopy and mass spectroscopy. The purity of the final product was estimated to be in excess of 99%.

Preparation of NTA-DTDA liposome suspensions

For NTA-DTDA incorporation into cells, dessicated NTA-DTDA was suspended to a concentration of 0.5 mM in PBS containing 0.5 mM Zn^{2+}, by sonication using a TOSCO 100W ultrasonic disintegrator at maximum amplitude for 2 min. The same procedure was used to produce suspensions of DMPC, and mixtures of NTA-DTDA and DMPC, POPC, or DOPE. Stock suspensions of lipids were stored at -20°C, and were always re-sonicated and diluted to the indicated concentration prior to use in experiments.

Monoclonal antibodies

The monoclonal antibodies (mAb) and their sources were as follows: murine anti-CD40 (clone 3/23, Rat IgG_{2a}) and murine anti-CD3 (clone 145-2C11, Armenian Hamster IgG) mAbs were both obtained from Pharmingen; and murine anti-B7.1 (clone 16-10A1, Armenian Hamster IgG) mAb was kindly provided by Dr. K. Shortman, WEHI, Melbourne. Where indicated, mAbs were biotinylated by reacting with sulfo-LC-biotin (Pierce) as previously described (26).

Recombinant proteins

Recombinant forms of the extracellular regions of the murine T cell co-stimulatory molecules B7.1 (CD80) and CD40, and the extracellular region of the human erythropoietin receptor (EPOR), each with a hexahistidine (6His) tag and denoted B7.1-6H, CD40-6H, and EPOR-6H, respectively, were produced using the baculovirus expression system. Briefly, genes encoding the extracellular domains of murine B7.1, CD40 and EPOR were amplified by polymerase chain reaction (PCR), and the sequences for 6His tags were incorporated into the end of each gene (corresponding to the carboxyl terminal of the protein) by PCR using primers containing the sequence of the tag. The constructs were then separately ligated into the pVL1393 plasmid baculovirus transfer vector and used to transform *E. coli.* Appropriate transformants were selected, and recombinant pVL1393 plasmids from these transformants were co-transfected with the baculovirus AcMNPV into SF9 insect cells. Cells infected with virus which had the pVL1393 plasmid incorporated into the viral genome were selected by plaque assays, further amplified and these viral stocks were used to infect High-5 insect cells grown in Express-5 medium. Recombinant proteins were purified from the supernatants of recombinant virus infected High-5 cells by Ni^{2+}-NTA affinity chromatography (using Ni^{2+}-NTA Superflow, from QIAGEN Pty Ltd, Cifton Hill, Victoria, Australia) followed by size exclusion gel filtration on FPLC (Pharmacia Biotech, Upsalla, Sweden) using a Superdex-75 HR 10/30 column; the final purity of each protein was >95% as judged by SDS-PAGE analysis. For some experiments recombinant proteins were biotinylated by reacting with sulfo-LC-biotin (Pierce) as previously described (26). The proteins were routinely stored at -20°C in PBS at a concentration of 0.2-0.6 mg/ml, and then thawed at 37°C and vortexed gently prior to use in each experiment.

Incorporating and optimizing the incorporation of NTA-DTDA

Cultured P815 tumor cells were washed twice in PBS to remove proteins from the culture media and suspended to 1 x 10^7 cells/ml in PBS. The cells were then aliquoted into 96-well V-bottom Serocluster plates (Costar, Corning, NY) at 1.8 x 1^5 cells/well and incubated with 125 μM NTA-DTDA (alone or as a mixture with other lipids as indicated) or 125 μM DMPC (control) in PBS containing 125 μM Zn^{2+}, for 40 min at 37°C. Following the incubation, unincorporated lipid was removed by washing three times with PBS containing 0.1% v/v BSA (PBS-0.1% v/v BSA). The relative level of NTA-DTDA incorporated was routinely assessed by FACS analysis (see below) after incubating the cells with biotinylated 6His peptide (B-6His) (0.2 μg/ml) for 30 min at 4°C, washing twice with PBS-0.1% v/v BSA, and then staining with streptavidin-FITC. The cells were incubated with streptavidin-FITC (33 μg/ml) in PBS containing 1% v/v BSA (PBS-1% v/v-BSA) for 30 min at 4°C, washed three times with PBS-1% v/v BSA, fixed with 2% v/v paraformaldehyde in PBS, and then analyzed for FITC-fluorescence by FACS.

To promote fusion of NTA-DTDA liposomes and hence incorporation of the NTA-DTDA into the membrane of cells, a number of agents previously reported to potentiate the fusion of cells and vesicles with lipid layers were tested. P815 cells aliquoted into 96-well V-bottom serocluster plates as described above were incubated with 125 μM NTA-DTDA, DMPC, POPC, or DOPE, or with 125 μM NTA-DTDA plus DMPC, POPC or DOPE (at the indicated molar ratio), in PBS containing 125 μM Zn^{2+}, for 40 min at 37°C. For some experiments the cells were treated with PEG following the incubation: the cells were pelleted, suspended in 15% PEG_{400}, mixed and diluted 10x with serum-free EMEM, and then washed once with serum-containing EMEM and twice with PBS-0.1% v/v BSA, before engrafting the cells with biotinylated recombinant protein (see below) and then staining with streptavidin-FITC as above for FACS analysis.

Engrafting recombinant proteins onto cells

Cells with incorporated NTA-DTDA were incubated with purified B7.1-6H and CD40-6H

(or biotinylated forms of these as indicated), either alone (each at 50 μg/ml) or in combination (100 μg/ml total protein, with a B7.1-6H:CD40-6H molar ratio of 4:1), in 96-well V-bottom Serocluster plates for 1 hr at 4°C. Unbound protein was then removed by washing twice with PBS-0.1% v/v BSA, before using the cells bearing the engrafted protein(s) either for immunizations or in assays of T cell proliferation. For experiments to determine the level of bound protein by FACS analysis the cells were stained with streptavidin-FITC (for cells bearing engrafted biotinylated protein), or were first incubated with the appropriate biotinylated mAb (B-mAb) (4°C for 30 min), washed twice with PBS-0.1% V/v BSA and then stained with streptavidin-FITC.

Time courses

Cells with incorporated NTA-DTDA and DMPC, with or without engrafted CD40-6H, were suspended in EMEM containing 10% v/v FCS and 50 μM added Zn^{2+}, and incubated in 12-well flat-bottom tissue culture plates (Linbro, ICN Biomedicals Inc, Aurora, OH) for approx. 2 min (time 0), or 4 or 24 hrs at 37°C. After the indicated incubation time, cells were collected from the 12-well flat-bottom plates, transferred to 96-well V-bottom Serocluster plates and washed twice in PBS-0.1% v/v BSA, before either staining with streptavidin-FITC (for cells with NTA-DTDA and engrafted B-CD40-6H), or first incubating with B-CD40-6H and then washing with PBS-0.1% v/v BSA and staining with streptavidin-FITC (for cells with only NTA-DTDA).

Flow cytometry

Fluorescence-activated cell sorter (FACS) analysis was used to quantify the relative levels of NTA-DTDA incorporated into the membrane of cells following binding of B-6His, and the levels of biotinylated recombinant proteins anchored to the cell surface *via* the incorporated NTA-DTDA. Flow cytometric analyses were performed using a FACSort flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 mW argon-ion laser. Cells were analysed on the basis of forward light scatter (FSC), side light scatter (SSC) and FITC-fluorescence; with the relative shift in fluorescence intensity above background providing a

semi-quantitative measure of the level of NTA-DTDA incorporation and the level of peptide or recombinant protein on the surface of cells. Typically, fluorescence information for 10, 000 cells was collected for each condition using a log amplifier and the data processed using CELLQuest (Becton Dickinson) software. Data were analyzed by gating live cells, as judged by FSC versus SSC dot plots, and plotting the fluorescence profile as a histogram. The fold increase in fluorescence intensity above background was determined by measuring the shift in fluorescence intensity, using the control sample as background, from peak to peak. The results of independent experiments were then represented as the mean ± the standard error of the mean (SEM).

Confocal microscopy

The distribution of the NTA-DTDA on the surface of P815 cells was studied by laser scanning confocal microscopy using cells bearing incorporated NTA-DTDA engrafted with biotinylated CD40-6H, and stained with streptavidin-FITC. Briefly, the cells were suspended in embedding medium (2% propyl gallate in 87% v/v glycerol) and deposited into 0.05 mm deep chambers on microscope slides formed using perforated Scotch 465 adhesive transfer tape, and the chambers were then sealed with glass cover slips. The cells were examined for fluorescence at 520 nm with a MRC-500 Laser Scanning Confocal Imaging System (BioRad), consisting of a Nikon confocal fluorescence microscope (x 60 Nikon objective), with a BioRad UV-laser scanner and an Ion Laser Technology laser head (model 5425, BioRad) with an argon ion laser. The image was acquired by Kalman averaging of 10 successive laser scans, and stored and analyzed using Image Processor PC (BioRad) and processed using NIH Image 1.61 software.

T cell proliferation assays

Murine T cells for use in T cell proliferation assays were isolated and purified from the spleens of either allogeneic or syngeneic mice as described (27). Briefly, the spleens were dissociated into single cell suspensions, and dead cells and red blood cells were removed by density gradient centrifugation using an Isopaque-Ficoll gradient. After centrifugation (20

min at 400 x g) the viable cells, mainly lymphocytes, were collected from the layer at the top of the gradient and suspended in RPMI 1640 containing 10% v/v FCS, 5 x 10^{-5} M 2-β-mercaptoethanol, 100 i.u./ml penicillin, 100 μg/ml neomycin, IL-2, and 10 mM HEPES. T cells were purified using an equilibrated nylon wool column (28). The purified T cells were then suspended in growth medium at a concentration of 2 x 10^4 cells/50 μl/well in a 96-well flat bottom plate (Cell Wells, Corning, NY) for culture at 37°C in an atmosphere of 5% CO_2.

T cell proliferation assays were carried out as described (28). Syngeneic lymphocytes or responder cells were then co-cultured with γ-irradiated (5000 rad) stimulator cells at a concentration of 2 x 10^4 cells/50 μl/well. Stimulator cells included native P815 tumor cells, P815 cells with incorporated NTA-DTDA on their surface, and P815 cells with engrafted recombinant protein(s), as indicated. After 4 days co-culture at 37°C, the cells were pulsed with 1 μCi of [^{3}H]-thymidine per well for 6 hrs. The cells were then harvested using a Filtermate 196 cell harvester (Packard) and [^{3}H]-thymidine incorporation assessed using MicroScint scintillant and a Topcount NXT microplate scintillation counter (Packard) using Topcount software.

Cytotoxicity assays

Assays for *in vivo* tumor-specific CTL were performed by a procedure similar to that described by Chen *et al.* (29). Syngeneic DBA/2 mice were immunized subcutaneously with either PBS (control) or 1 x 10^5 γ-irradiated (5000 rad) P815 cells engrafted with recombinant protein(s). Spleens were removed from mice 14 days after immunization, and T lymphocytes (effector T cells) were isolated by density gradient centrifugation using Isopaque-Ficoll and nylon wool fractionation, as described above. Effector T cells were then suspended in incubation medium and aliquoted into 24-well flat-bottom plates at a concentration of 1 x 10^5 cells/well and co-cultured with 1 x 10^5 γ-irradiated (5000 rad) native P815 cells. After 5 days of co-culture, the cytolytic activity of the effector cells was assessed in a standard ^{51}Cr-release assay, as described (29). Briefly, 2 x 10^6 native P815 cells were labelled with 250 μCi ^{51}Cr ($Na^{51}CrO_4$) for 90 min. Labelled target cells were washed three times and resuspended in culture medium. Effector and target cells were co-incubated

with effector cells at different effector to target ratios, as indicated, for 6 hrs at 37°C. Supernatants were harvested and ^{51}Cr release assessed with a Topcount NXT microplate scintillation counter (Packard) using Topcount software (Packard). Percent specific lysis was calculated as follows:

$$\%\,\text{Specific lysis} = \frac{100 \times (\text{experimental cpm} - \text{spontaneous cpm})}{\text{maximal cpm} - \text{spontaneous cpm})}$$

Immunization of animals and tumor challenge in vivo

Mice were immunized using a protocol similar to that described (29). Briefly, either PBS (control) or 1 x 10^5 γ-irradiated P815 cells with the engrafted recombinant protein(s) as indicated, were suspended in a 0.2 ml volume of PBS and injected into the shaved right back of syngeneic DBA/2 mice using a 25-gauge needle and 1 ml syringe. After 14 days the mice were either used in cytotoxicity assays using T cells isolated from the spleens of the mice, or were challenged with 1 x 10^5 native P815 cells by subcutaneous injection in the shaved left back. For monitoring tumor growth, the mice were scored for tumor size once a week by measuring two perpendicular diameters in millimeters using a caliper (29). Survival data represent animals that were still alive when scored; animals that were near death were euthanized after scoring and were deemed to have died of the tumor. Data for a total of 10 or 12 mice in the group for each experimental condition is presented.

EXAMPLE 1

The IAsys biosensor can be used to study the interaction of hexa-histidine-tagged proteins anchored onto the lipid layer.

A planar membrane layer consisting of PC and PE-NTA was formed on the sensing surface of the IAsys biosensor cuvette. Figure 2(a) shows the refractive index change due to formation of the layer (indicated Memb); there is no signal following two lots of washings with TBS (as marked), indicating that a stable layer is formed. Subsequently, the biosensor showed no change in refractive index on addition of BSA (see Figure 2(b)), indicating a low level of non-specific binding. However, the addition of a 13 kDa transcription factor with hexa-histidine tag (denoted by HH in Figure 2(b)) resulted in a strong signal indicating binding of this protein to the NTA-PE in the membrane *via* the NTA chelating linkage. The anchored protein was not removed after washing and incubating in TBS, but was removed

with 200 mM imidazole known to break the chelating linkage (denoted Imid+TBS). Moreover, the interaction of other molecules with this protein also could be readily detected. The results show that hexa-histidine-tagged proteins can be stably and effectively anchored onto the layer to study molecular interactions with the biosensor.

EXAMPLE 2

Molecules anchored onto the lipid layer can diffuse laterally and interact.

A microscope slide was reacted with octadecyltrichlorosilane to form an octadecane monolayer (as described above). A phospholipid layer was then formed on the monolayer by placing a drop of the micelle (e.g. liposome) suspension (PC and biot-PE, 10:1) and incubating for 2 hours. After washing, the surface of the layer was stained with fluoresceinated streptavidin, incubated with Biot-BSA at 4°C, and then examined for fluorescein fluorescence. Preliminary studies show that under these conditions the fluorescence is seen uniformly over the surface. However, after a 30-60 minute incubation of the slide at 37°C the fluorescence is seen in discrete patches, indicating that the fluoresceinated streptavidin forms aggregates with the biot-BSA. This shows that, depending on the temperature, molecules on the layer can diffuse laterally and interact.

EXAMPLE 3

Using the technique of this invention to resolve current difficulties in understanding of the CD4-MHC Class II interaction.

One application of the invention described herein is to study the interaction of CD4 with Major Histocompatibility Complex (MHC) Class II molecules, two key molecules involved in initiating an immune response by white blood cells. Crucial for the initiation of an antigen-specific immune response is the interaction of CD4 with invariant regions on the MHC Class II molecule, which provides adhesion and stabilizes the interaction between the T cell receptor and the presented antigen. Although cellular immunology experiments

strongly suggest that such an interaction is crucial for efficient T cell activation and the triggering of an immune response, to date no direct evidence of this interaction or how it takes place, has been obtained.

Recent X-ray crystallographic studies on the three-dimensional structure of the extracellular portion of the human MHC Class II (HLA-DR1) molecule have shown that this molecule crystallizes as a dimer of non-covalently associated heterodimers. This suggests a capacity of the MHC Class II to dimerize, perhaps leading to an increased avidity for the CD4 receptor. The crystal structure of human soluble CD4 has shown that the D1-D2 and the D3-D4 regions of CD4 crystallize in multimeric form; but the affinity of self-association must be low because multimers do not form in solution. It has been suggested, that the extreme concentration of soluble CD4 in the crystal may reveal a natural propensity for oligomerization. Interestingly, biochemical evidence indicates that some CD4 immunoprecipitates as a disulfide-linked homodimer from lysates of sheep lymphocytes. Recently, evidence that human CD4 can oligomerize also was obtained using CD4 chimeras in transfectants, and by chemically cross-linking adjacent CD4 molecules on the cell surface. However, these findings do not clarify whether oligomerization is essential to increase the avidity of the CD4-MHC Class II interaction. Application of the technique as described below will provide the means to redress this deficiency.

EXAMPLE 4

The aim of this example is to determine whether the extracellular regions of mouse and human CD4 can oligomerize when anchored onto membrane layers that allow lateral CD4 mobility.

The atomic force microscope can be used to study the oligomerization of CD4 when anchored onto a lipid layer. A phospholipid layer consisting of 90% PC and 10% PE-NTA can be formed on a freshly cleaved and atomically flat surface of mica to permit high resolution atomic force microscopy. The procedure for this is the same as that used for forming layer on the biosensor surface (see above). Alternatively, the surfaces of glass-like

materials, or surfaces coated with a uniform layer of gold (400-1000 A thick) using sputtered techniques, can be suitably treated with compounds like octadecyltrichlorosilane and octadecanethiol, respectively, to form a hydrophobic monolayer of hydrocarbon chains covalently attached to the surface and capable of reacting with a suspension of micelles (e.g. liposomes) to produce a hybrid layer consisting of the attached monolayer of octadecane hydrocarbon chains and the phospholipid layer comprised of PC/PE-NTA.

The recombinant extracellular region of CD4 with hexa-histidine tail can be anchored using the NTA chelating linkage onto layers formed on the glass or mica surface suitable for use in the atomic force microscope. The particular PC used to produce the PC/PE-NTA membrane layer can be chosen with chain lengths such that the membrane transition temperature is about 23°C (eg. dimyristoylphosphatidylcholine). Atomic force measurements can then initially be carried out with the system maintained at 10°C to prevent CD4 lateral mobility to map the force structure with CD4 in the monomeric state. The temperature can then be increased to 37°C for 30-60 minutes to allow CD4 to diffuse and interact laterally, before again cooling the system to 10°C to map the structure. Comparison of three-dimensional force maps of the CD4 molecules under the different conditions should allow determination of whether murine CD4 can oligomerize.

The IAsys biosensor can be used to measure the binding affinities (association and dissociation constants) for the CD4-MHC Class II interaction at 10°C and 37°C, using CD4 anchored onto layers formed on the sensing surface of the biosensor cuvette. The finding of a higher binding affinity for the interaction of CD4 with soluble MHC Class II after subjecting to temperatures above the membrane transition temperature would provide further evidence that the extracellular region of CD4 has oligomerized. These experiments initially can be carried out using murine CD4 and MHC Class II with and without bound peptide. Similar experiments can be carried out using anchored human CD4 and soluble human MHC Class II. The force structure of CD4 anchored onto layers under conditions expected to exist in oligomeric form also can be mapped after binding soluble MHC Class II, if the interaction is stable to permit analysis, to see if MHC Class II binding can induce changes in the structure.

EXAMPLE 5

The aim of this Example is to determine whether the extracellular regions of mouse and human MHC Class II oligomerize when anchored onto layers that allow lateral MHC Class II mobility.

Analogous to the studies described above for CD4, the atomic force structure of murine MHC Class II when anchored onto the layers can be mapped with the system maintained at 10°C, and compared with that observed after exposure to 37°C, to see if oligomerization occurs. The studies also can be carried out with and without peptide bound to the MHC Class II groove (for the mouse system), to determine if bound peptide has an effect on MHC Class II oligomerization. The atomic force structure also can be mapped after binding soluble murine CD4 to see if this can induce changes in the force structure and/or promote MHC Class II oligomerization. The biosensor can be used with membrane-anchored MHC Class II to measure the binding affinities of the interaction with soluble CD4, under conditions in which the MHC Class II is expected to exist in either monomeric or oligomeric form. The finding of a higher binding affinity of the MHC Class II for soluble CD4 after subjecting the system to temperatures above the membrane melting temperature, may provide additional evidence that MHC Class II can oligomerize.

EXAMPLE 6

The aim of this Example is to test the hypothesis that oligomerization of CD4, and oligomerization of MHC Class II, are both required for CD4-MHC Class II interaction.

Failure to detect binding of either monomeric or oligomeric CD4 to soluble MHC Class II, and either monomeric or oligomeric MHC Class II to soluble CD4 in the experiments outlined above could indicate that oligomerization of CD4 and MHC Class II are both necessary for stable interaction. If both CD4 and MHC Class II can be shown to oligomerize in the membrane system, this possibility can be tested by examining whether CD4 and MHC Class II are able to interact under conditions where both are expected to exist in oligomeric

form. This can be done by anchoring hexa-histidine-tagged MHC Class II onto unilamellar vesicles using the NTA chelating linkage, expose the vesicles to 37°C to permit MHC Class II oligomerization, and study the ability of these vesicles to interact with oligomeric CD4 using the IAsys biosensor. This can be done in both the mouse and human systems, but since the mouse MHC Class II with and without peptide in the groove is available, the mouse MHC in the presence of bound peptide may be required to achieve the correct molecular confirmation required for the interaction.

The feasibility of this approach has been demonstrated recently by studies showing the interaction of biotin-PE and PC-containing vesicles with strepavidin anchored onto a lipid layer formed on the IAsys biosensor surface. Figure 3 is a biosensor trace showing changes in refractive index due to additions made to an IAsys biosensor cuvette after reacting the sensing surface of the cuvette with a suspension of vesicles consisting of PC and biot-PE. The initial change in refractive index is due to the formation of the layer. Subsequently, the cuvette was washed with Tris buffer. After equilibration, the addition of BSA (indicated BSA) resulted in no change in refractive index, indicating a low level of non-specific binding. The addition of streptavidin (indicated Strep) resulted in a change in refractive index, which did not alter upon washing with Tris buffer (indicated Tris). The addition of a suspension of vesicles made of PC (indicated PC) did not bind to the membrane, but the addition of vesicles made of PC and biot-PC did interact with the membrane, reflecting the binding of the vesicles containing the biot-PE with the streptavidin anchored onto the membrane.

EXAMPLE 7

Since the Examples above use recombinant forms of the extracellular regions of receptor molecules oligomerization would be detected only if it arises from interactions *via* the extracellular region of the molecules. For many receptors this is unlikely to present difficulties as both the transmembrane and intracellular regions are short and are unlikely to be involved in oligomerization. Certainly, for the CD4 and MHC Class II described above, this notion also is supported by X-ray crystallographic studies which show that the

extracellular domains of the molecules each can dimerize/oligomerize spontaneously in the absence of other molecules (e.g. those in the cytoplasm). However, other applications of the technique also include the anchoring of the cytoplasmic or transmembrane domain of receptor molecules onto the model membranes to study their involvement in oligomerization and/or in interactions with other membrane associated or cytoplasmic molecules involved in signal transduction.

Another useful application of the instant technology is to study the interaction of CD2 with its ligands. Thus, the use of multimeric binding techniques have identified the glycosyl-phosphatidylinositol (GPI) anchored molecule CD59 as an alternative ligand for human CD2, but biosensor studies have failed to confirm the interaction of CD2 with monomeric soluble CD59. This indicates that this interaction is of low affinity and that oligomerization of CD59 may be required to form a stable interaction with CD2. Clearly, analogous to the technique used above for studying CD4-MHC interactions, the anchoring of CD59 onto lipid layers coupled with biosensor and atomic force analysis could be extremely useful in determining whether oligomerization of CD59 is required for interaction with CD2. This technique also is particularly suited in applications for studying the interactions of other GPI-anchored receptor molecules. Such receptor molecules could be anchored to the membrane either directly through their native GPI anchor, or by engineering a hexa-histidine tag to replace the native GPI moiety, and using the hexa-histidine-NTA chelating linkage to anchor the receptors onto the membrane.

The Examples described herein establish that the technique in conjunction with the technologies outlined is very useful for the analysis of other low affinity molecular interactions. The subject technology can also be used to assay for interactions or aggregation events between receptor domains, proteins, glycoproteins, polysaccharides, or fragments thereof, after attachment of an appropriate hexa-histidine tag and the anchoring of the molecules onto the layer membrane system. This provides a method of screening for agents or drugs that may influence such receptor interactions. The invention therefore, provides the basis of a novel method of screening for drugs or other agents that influence receptor interactions, and consequently, the ability of the receptors to transduce transmembrane

signals and/or to elicit responses in biological systems.

EXAMPLE 8

Using the instant invention to anchor or engraft hexa-histidine tagged molecules onto the surfaces of cells and other biological and synthetic membranes (see Figure 4), for the development of vaccines and for drug targeting.

The histograms in Figure 5 show fluorescence-activated cell sorting (FACS) profiles of murine mastocytoma P815 cells carrying engrafted recombinant hexa-histidine-tagged murine B7.1 and CD40. P815 cells were pre-incubated for 30 min at 37°C with a suspension (0.1 mM) of control lipid di-myristoyl-phosphatidylcholine (DMPC; also referred to as di-C14-PC; control), or the chelator lipid NTA-DTDA, before being washed in PBS and incubated with a mixture of hexa-histidine-tagged B7.1 and CD40 (each at ~20 mg/ml). The cells were then washed again in PBS and stained by an incubation (30 min at 4°C) with either biotinylated 16-10A1 or biotinylated B-3/23 monoclonal antibody (ie. biotinylated anti-B7.1 or anti-CD40), as indicated, followed by an incubation with FITC-conjugated streptavidin. Cells incubated with DMPC and recombinant proteins show a low level of fluorescence after staining with either monoclonal antibody (Control). The fluorescence of P815 cells pre-incubated with NTA-DTDA is 10-100-fold higher than that of cells pre-incubated with DMPC (Control). Each result is a representative of two experiments performed in duplicate. The results show that chelator lipids (in this instance NTA-DTDA) can be incorporated into the membrane of these cells, and that the incorporated lipid can be used to anchor or engraft hexa-histidine tagged B7.1 and CD40 directly onto the P815 cell surface *via* the NTA-DTDA. In other studies we showed that recombinant murine B7.1 and CD40 bearing a hexa-histidine tag can be engrafted onto the surface of all the different cell lines tested; these included murine P815 and EL4 tumor cells, human leukemic Jurkat cells and yeast cells.

EXAMPLE 9

The Example relates to modifying the surface of tumor cells to enhance tumor immunity.

Recent work indicates that the transmembrane and cytoplasmic regions of B7-1 and B7-2 are not required for T cell co-stimulation (23), and that T cell co-stimulation also occurs when the B7-1 is expressed on tumor cell surfaces in a GPI-anchored form (24). Also, the extracellular regions of any cell surface receptor molecules (e.g. the murine T cell co-stimulator molecules B7.1 and CD40) can be produced to contain a hexa-histidine or other appropriate peptide tag on the carboxyl terminal. In this form the present invention provides a method of anchoring these co-stimulator molecules directly onto the cell surface in the correct orientation, thereby mimicking the co-stimulatory function of these molecules on the surface of antigen presenting cells. The instant invention, therefore, has implications for tumor vaccine development, by providing a more convenient and safe alternative to transfection for putting co-stimulator and/or other relevant molecules onto tumor cells for use in immunizations to enhance immunity to tumors.

The viability of using engrafted molecules can be tested by assaying for functional responses dependent on the engrafted molecules. Thus, the ability of murine P815 mastocytoma (DBA/2, H-2d) cells carrying engrafted hexa-histidine tagged B7-1 and/or CD40 to stimulate a T cell proliferative response in an allogenic system was examined using splenocytes isolated from C57Bl/6 (H-2b) mice co-cultured with an appropriate number of γ-irradiated P815 cells (as control), or P815 cells engrafted with hexa-histidine-tagged B7-1 and/or CD40. Preliminary experiments in which the incorporation of 3H-thymidine was used to measure T cell proliferation, show that the P815 cells bearing engrafted hexa-histidine tagged B7-1 and/or CD40 are able to stimulate an increased level of T cell proliferation in this mixed cell reaction. These results are consistent with the invention being useful to modify cells for use in vaccinations to enhance anti-tumor responses.

EXAMPLE 10

To test the ability of P815 cells bearing engrafted co-stimulatory molecules to induce anti-tumor responses *in vivo*, mice were immunized with P815 cells bearing the engrafted

molecules to see if this could stimulate CTL activity and/or affect tumor growth in syngeneic animals. Separate groups of DBA/2 mice were immunized with either PBS, or with γ-irradiated P815 cells bearing engrafted EPOR-6H, B7.1-6H, or B7.1-6H plus CD40-6H. Two weeks after immunization, spleens were removed from the mice, and splenic T cells were isolated and assessed for their ability to kill native P815 cells in a standard ^{51}Cr release assay. The data in Figure 6 show that at all the effector target cell ratios indicated (0.5:1, 1:1, 5:1), only a low level (2-5%) of lysis was induced by T cells from mice immunized with PBS (as control). The lytic activity of T cells from mice immunized with P815-EPOR (as control protein) was also low ranging from 7-16%. Interestingly, at all effector:target cell ratios tested, the level of tumor cell-specific lysis was higher for conditions where the effector T cells were derived from mice immunized with P815 cells bearing one or more engrafted co-stimulatory molecule(s) (see Figure 6). The highest cytolytic activity was observed at the effector:target cell ratio of 5:1, for which the specific lysis induced by T cells obtained from mice immunized with P815 cells bearing engrafted B7.1, and P815 cells with engrafted B7.1 and CD40, was 3- and 5-fold higher, respectively, than that for T cells obtained from mice immunized with P815 cells engrafted with control protein (see Figure 6). Parallel experiments using native EL4 cells instead of P815 cells showed only background levels of lysis, indicating that the cytolytic response was specific for P815 cells as targets. The results indicate that CTL responses against P815 cells can be generated in mice immunized with P815 cells bearing engrafted B7/CD40.

To determine whether the immunization of mice with P815 cells bearing engrafted co-stimulatory molecules could induce tumor immunity, groups of mice immunized with γ-irradiated cells bearing the engrafted proteins, also were monitored for tumor growth and survival after a challenge with native P815 cells. These studies indicated a slower rate of tumor growth in mice immunized with P815 cells bearing engrafted co-stimulatory molecule(s), compared to mice immunized with cells bearing control protein. Thus, at 5 weeks after tumor challenge the mean tumor diameter was 3.36 ± 1.0 mm and 1.1 ± 0.9 mm, for mice immunized with P815 cells bearing engrafted B7.1-6H and B7.1-6H plus CD40-6H, respectively; and 10.7 ± 2.5 mm and 8.3 ± 2.7 mm for mice immunized with PBS and P815 cells engrafted with EPOR-6H, respectively. Tumor growth data as reflected by the

mean tumor diameter for only the first 5 weeks after challenge is presented since from this time some animals died from the tumor. At 14 weeks after tumor challenge survival was ~17% for control mice, ~30% for mice immunized with P815 cells engrafted with B7.1, and ~60% for mice immunized with P815 cells engrafted with both B7.1 and CD40 (see Figures 7(a) and (b)). Consistent with the observed increase in CTL activity, the results indicate that the immunization of syngeneic animals with P815 cells bearing engrafted co-stimulatory molecules can inhibit tumor growth and prolong survival of the animals after a challenge with the native P815 tumor.

EXAMPLE 11

Vascular endothelial growth factor (VEGF) is a homodimeric glyocoprotein hormone of ~40 kDa that is among the most potent of angiogenic mitogens, and a major regulator of angiogenesis (30). Considerable evidence suggests that VEGF is secreted by tumor cells and other cells exposed to hypoxia, and that VEGF is a major angiogenic factor in solid tumor development (31). In addition to its potent angiogenic activity, VEGF increases vascular permeability and favours the migration of endothelial cells through the extravascular matrix, processes that are essential for tumor angiogenesis, tumor spread, and metastasis (32). Human VEGF is known to stimulate endothelial cell growth and differentiation by binding to high affinity VEGF receptors such as the kinase domain receptor (human KDR, or murine flk-1), and the Fms-like tyrosine kinase-1 (Flt-1). These receptors are expressed exclusively on proliferating vascular endothelial cells, and their expression is known to be increased by a number of factors often produced by tumors (30-33). That VEGF and its receptors are important for tumor growth has been demonstrated by the fact that the neutralization of VEGF by the use of antibodies (34) or recombinant soluble receptor domains (35) exhibit therapeutic potential as agents that can suppress tumor growth and metastasis *in vivo*.

The substantially exclusive expression of VEGF receptors on proliferating endothelial cells, suggests the VEGF receptor can be used as a targeting molecule in therapeutic strategies that target neovascularization. The development of sterically stabilized or "stealth" liposomes (SLs) which can evade elimination by the phagocytic cells of the immune system (e.g. the

reticulo-endothelial system in the liver and spleen), recently has provided a major advance for liposomal drug delivery in cancer chemotherapy (36-38). In contrast to conventional liposomes which are cleared rapidly from the blood (often within minutes) after their administration *in vivo*, SLs remain in the blood circulation for several days. A number of studies have demonstrated therapeutic benefit of SLs containing an encapsulated cytotoxic drug like doxorubicin, in the treatment of AIDS-related Kaposi sarcomas and other lesions characterized by leaky vasculature (38). Recent studies also describe the targeting of SLs to specific tumors, with the targeting being achieved primarily by the use of "immuno-liposomes", or liposomes with specific antibody (or $F(ab')_2$ fragments) covalently attached to the liposome surface (39). The immobilization of targeting proteins such as antibodies onto SLs encapsulated with doxorubicin apparently does not alter their stealth-like characteristics, but can endow the liposomes with specific targeting properties (40-43).

Until the advent of the present invention, the coupling of target molecules to liposomes has been difficult and there is potential to induce unwanted anti-idiotypic responses to the antibody used. In accordance with an aspect of the present invention, two chelator lipids, nitrilotriacetic acid di-tetra-decylamine (NTA-DTDA) and nitrilotriacetic acid polyethyleneglycol (2000) phosphatidylethanolamine (NTA-PEG2000-PE), are used with a recombinant form of VEGF, to develop SLs containing encapsulated doxorubicin that will target and specifically destroy proliferating vascular endothelial cells *in vivo*, thereby blocking neovascularization and tumor growth.

VEGF is a particularly attractive targeting molecule as VEGF receptors are expressed substantially exclusively on angiogenic endothelium. In accordance with the present invention, it is proposed to produce a "stealth" liposome containing encapsulated doxorubicin and surface VEGF anchored through NTA-lipids like NTA-DTDA. It is proposed in accordance with the present invention that when administered *in vivo*, this agent will target and destroy proliferating endothelial cells, and will inhibit tumorigenesis and tumor metastasis.

Liposomes composed largely of conventional lipids like egg-yolk phosphatidylcholine (PC)

and cholesterol (Chol), and a small proportion (~10%) of a sterically "stabilizing" lipid such as ganglioside GM1 (35), or a phosphatidyl-ethanolamine conjugated to polyethyleneglycol (2000) (PEG2000-PE) (36, 37), are reported to exhibit increased stability and prolonged circulation times in blood, largely escaping elimination by the reticulo-endothelial system. These properties of SLs have been attributed to the presence of lipids possessing uncharged headgroups which increase interaction with water, but inhibit interaction with either charged or hydrophobic structures likely to be encountered on proteins and cells in plasma (35, 36). Evidence suggests that SLs (or immunoliposomes) with antibody molecules attached to the distal end of the PEG chains on the SL surface, interact more effectively with their target than liposomes with the antibody attached directly onto the SL surface. This has been explained by the PEG chains sterically interfering with the ability of the antibody to interact with antigen under these conditions (40). SLs made using ganglioside GM1, or a PEG-lipid with a shorter PEG chain length (eg. PEG750-PE, rather than PEG2000-PE), therefore, are likely to be more suited for the binding of 6His-VEGF directly to the NTA-DTDA on the liposome surface, and for optimal binding of the engrafted VEGF to VEGF receptors on target cells. Ganglioside GM1 and PEG750-PE are both commercially available (from Avanti Polar Lipids), and each will be tested with liposomes made from PC, Chol and NTA-DTDA. To further reduce possible steric effects the inventors produce a novel lipid, namely NTA-PEG2000-PE, which consists of the NTA group attached to the distal end of the PEG chain on the PE. This compound, is used in combination with PEG2000-PE to produce SLs which allow convenient engraftment of targeting 6His-proteins (such as 6His-VEGF), while eliminating the possibility of steric hindrance. This is approach greatly facilitates the use of SLs in therapeutic applications requiring their targeting to specific cells and/or tissues.

Recombinant 6His-VEGF is produced. SLs are produced from a mixture of lipids including PC, Chol, NTA-DTDA, and "stealth" lipids such as ganglioside GM1, PEG2000-PE and NTA-PEG2000-PE. The SLs are engrafted with 6His-VEGF (VEGF-SLs) and then assessed for their ability to target endothelial cells. Conditions for specific binding of the liposomes to proliferating endothelial cells in culture are optimized, relative to their binding to cells that lack the VEGF receptor. The specific cytoxicity of VEGF-SLs encapsulated with doxorubicin will be assessed using human vascular endothelial cells *in vitro*. VEGF-SLs

intrinsically labelled with fluorescent dyes and/or radioactive tracers are administered intravenously into mice to determine their distribution in various tissues with time. The proportion of each stabilizing lipid used for producing SLs is altered to optimize the "stealth" properties of the liposomes, as judged by a reduction in the proportion of the liposomes taken up by the liver, spleen and other major organs, relative to vascularizing tumors. As the VEGF receptor is endocytosed upon binding its ligand, VEGF-SLs made to contain encapsulated doxorubicin, are taken up by proliferating vascular endothelial cells, resulting in their destruction. The viability of this method is tested by examining the ability of the liposomes to inhibit tumor growth and/or to eradicate established tumors *in vivo*. This work provides therefore a novel approach to anti-angiogenic cancer therapy.

EXAMPLE 12

The method used to anchor proteins onto membranes is also used to engraft recombinant receptors onto cells which, when used, vaccines can modify immunological responses *in vivo*.

This is demonstrated by the fact that P815 cells engrafted with the co-stimulator molecules B7.1 and CD40 can be used as a vaccine to enhance tumor immunity. Analogously, these or any other recombinant protein or molecules (possessing the appropriate tag) may be engrafted onto any other biological membranous structure(s) (e.g. a membranous structure derived from cells and/or sub-cellular components, such as plasma membranes vesicles etc.). The engrafted structures can then be used as a vaccine to enhance tumor immunity and/or modify immunological responses *in vivo* for therapeutic purposes. The preferred method comprises:-

(i) incubating the cells or membranous material with a chelator lipid such as NTA-DTDA to allow the lipid to incorporate in the cells or membranes;

(ii) washing off any unincorporated lipid by centrifugation or filtration and resuspension of the structures in the appropriate solution or buffer;

(iii) incubating the membranous structures containing incorporated chelator lipid with an appropriate recombinant protein(s) possessing an appropriate affinity tag; and

(iv) washing off unincorporated protein material;

and then using the modified structure as a vaccine administrable *in vivo* for therapeutic purposes such as to modify immunological responses *in vivo*.

The subject approach can also be used with synthetic membrane structures (i.e. synthetic liposomes or vesicles composed of a mixture of any phospholipid (e.g. PC or PE) and the NTA-DTDA. The synthetic structures can be made to incorporate the NTA-DTDA, therefore, only steps (iii) to (v) above are required.

EXAMPLE 13

Preliminary experiments indicate that synthetic liposomes (composed of say PC and NTA-DTDA in 10:1 ratio) engrafted with an appropriate recombinant receptor protein can be used to specifically target cells bearing the cognate receptor or ligand (see Figure 8). Such liposomes also can be used to modify biological response (see Figure 9). The present invention provides, therefore, a method of engrafting recombinant proteins onto liposomes for use in therapeutic applications to deliver an encapsulated drug or other therapeutic agent to cells or tissues within the body. Such liposomes are used to modify a biological response(s) for the treatment of disease, or for targeting the delivery of cytotoxic drugs or agents to specific cells (e.g. tumor cells) in order to destroy such cells for therapeutic purposes.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in

this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS

1. A method of anchoring receptor domains onto a planar supported membrane comprising amphiphilic molecules arranged in a layer, wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group such that some of the metal chelating groups are oriented toward the outside surface of said membrane; which method comprises the step of interacting a receptor domain which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach to said membrane *via* the outwardly facing metal chelating residues of said membrane, such that the receptor domains are capable of lateral movement, dimerization or oligomerization, and interaction with a ligand molecule.

2. A method according to Claim 1 wherein the metal chelating group is nitrilotriacetic (NTA).

3. A method according to Claim 2 wherein the membrane is formed from a suspension of micelles or liposomes from the amphiphilic molecules.

4. A method according to Claim 2 wherein the membrane is formed by the addition of water or aqueous buffers to a solution of amphiphilic molecules in an organic solvent.

5. A method according to Claims 3 or 4 wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group.

6. A method of anchoring receptor domains onto a lipid membrane such that the receptor domains are capable of lateral movement, said method comprising of:

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the

molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of micelles (e.g. liposomes) from amphiphilic molecules wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group, then interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a membrane layer to form in which some metal chelating residues attached to the amphiphilic molecules of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the metal chelating linkage to the outwardly-facing metal chelating residues of said membrane.

7. A method according to Claims 1 or 6 wherein the amphiphilic molecules are surfactant molecules having a hydrophilic "head" portion and one or more hydrophobic "tails".

8. A method of anchoring receptor domains onto a lipid membrane such that said receptor domains are capable of lateral movement to facilitate interactions with biomolecules in the membrane, said method comprising of:

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating

group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of micelles (e.g. liposomes) from a first phospholipid and a second phospholipid wherein the second phospholipid has been modified by a covalent attachment of a nitrilotriacetic acid (NTA), and then interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a phospholipid membrane layer to form in which some NTA residues attached to the second phospholipid of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the NTA-metal chelating linkage to the outwardly-facing NTA residues of said membrane.

9. A method according to Claim 8 wherein the first phospholipid is phosphatidylcholine (PC) and the second lipid is phosphatidyl-ethanolamine-NTA (PE-NTA) and the ratio of PC:PE-NTA (w/v) is about 10:1.

10. A method according to any one of the preceding claims wherein the support is the sensing surface of an optical biosensor or a mica surface suitable for analysis by atomic force microscopy.

11. A method according to any one of the preceding claims wherein the polypeptide tag comprises a sequence of at least six amino acid residues.

12. A method according to Claim 11 wherein the six amino acid residues is hexa-histidine.

13. A method of assaying interactions between a receptor and a ligand, comprising the steps of:-

(A) anchoring a receptor to a membrane by:-

(i) forming a membrane on an appropriate planar support by either:

(a) contacting and incubating the support for a period of time with a solution in an organic solvent of amphiphilic molecules wherein a proportion of the molecules have been modified by covalent attachment of a metal chelating group, then forming a membrane by the addition of water or an aqueous buffer; or

(b) forming a suspension of micelles (e.g. liposomes) from amphiphilic molecules wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group, and then interacting said micelle (e.g. liposome) suspension with an appropriate planar support for a time and under conditions sufficient to allow a membrane layer to form in which some metal chelating residues attached to the amphiphilic molecules of said micelle (e.g. liposome) suspension are oriented toward the outside surface of said membrane; and

(ii) interacting the receptor domain(s) to be anchored which is covalently attached to a polypeptide tag with said membrane for a time and under conditions sufficient for said polypeptide tag to attach *via* the metal chelating linkage to the outwardly-facing metal chelating residues of said membrane;

(B) allowing said receptor molecules to interact and/or oligomerize on the membrane; and

(C) contacting said anchored receptor with an effective concentration of ligand for a time and under conditions sufficient for binding to occur and detecting said binding.

14. A method according to Claim 13 wherein the interaction and/or oligomerization of the receptor and/or binding to the ligand is detected by atomic force microscopy or by an optical biosensor technique, for use in assays of intermolecular interactions, receptor dimerization/oligomerization, or in devices for the screening of drugs which affect these processes.

15. A method of engrafting or anchoring molecules onto liposomes, said method comprising:-

(i) preparing a suspension of liposomes with chelator lipid incorporated, and with or without an encapsulated drug or agent;

(ii) incubating the liposomes with a recombinant protein or target molecule bearing an appropriate metal affinity tag; and

(iii) if necessary, removing excess protein by washing, filtering or other washing means and suspending them in an appropriate solution.

16. A method according to Claim 15 wherein the molecules engrafted, anchored or encapsulated within the liposome are therapeutic molecules, pharmaceutical compounds, DNA and/or RNA.

17. A method according to Claim 16 wherein the molecules engrafted or anchored onto the liposome surface is VEGF or its homologue.

18. A method according to Claim 17 wherein the liposome is encapsulated with a cytotoxic drug or agent together with the engrafted VEGF or its homologue to block the growth of new blood vessels needed for the growth of tumors.

19. A method according to Claim 16 wherein the liposome comprises an immunogenic agent and together with an agent which targets the liposome to different cell types in the body including immune cells and tumor cells to alter immunogenicity or immunological responses.

20. A method of anchoring a recombinant molecule directly onto biological membranes, said method comprising:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or biological membranous structures with a suspension of the chelator lipid to allow the chelator lipid to incorporate into the structures;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the membranous structures with a solution of recombinant protein or target molecule possessing an appropriate metal affinity tag; and

(v) washing away excess or unbound soluble protein, and suspending the structures in a solution suitable for administration *in vivo*.

21. A method according to Claim 20 wherein the recombinant molecule is a co-stimulatory molecule.

22. A method according to Claim 20 or 21 wherein the biological membrane is from a tumor cell.

23. A method according to Claim 20 or 21 or 22 for use in enhancing or

modifying immunity to tumors or other disease conditions.

24. A method according to Claim 20 wherein the recombinant molecule is a receptor or ligand.

25. A method according to Claim 24 wherein the recombinant molecule is a ligand for a receptor on specific cell types within the body or on cells such as tumor cells that arise as a consequence of disease.

26. A method for altering the immunogenicity of a target cell or membranous component thereof, said method comprising anchoring a molecule to the membrane of said target cell by:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or membranous structures with a suspension of the chelator lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the membranous structures with a solution of said molecule to be anchored; and

(v) washing away excess or unbound soluble molecule, and suspending the structures in a solution suitable for administration *in vivo*.

27. A method according to Claim 26 wherein the target cell is a tumor cell.

28. A method according to Claim 26 or 27 wherein the molecule is a ligand, receptor, recombinant protein, polysaccharide, glycoprotein or antigen.

29. A method of targeting a liposome or a biological membranous structure to a particular cell or tissue, said method comprising anchoring or engrafting a molecule having a binding partner on the particular cell or tissue to be targeted by:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or biological membranous structures with a suspension of the chelator lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the liposomes or membranous structures with a solution of molecules to be anchored; and

(v) washing away excess or unbound soluble molecule, and suspending the structures in a solution suitable for administration *in vivo*.

30. A method of treatment, said method comprising administering to a subject an effective amount of a liposome preparation or membranous material comprising an active material and optionally an anchored or engrafted molecule having a binding partner or target tissue.

31. A method according to Claim 30 wherein the active material is a recombinant polypeptide, co-stimulatory molecule, therapeutic drug or nucleic acid molecule, either engrafted onto the surface or encapsulated within the liposome or membranous material.

32. A method according to Claim 30 or 31 wherein the anchored or engrafted molecule is a receptor, ligand, glycoprotein, polysaccharide or recombinant polypeptide.

33. A method according to Claim 32 wherein the anchored molecule is VEGF.

34. A method according to any one of Claims 30 to 33 when used to enhance immunity to a specific tumor or disease.

35. A method according to Claim 31 wherein the co-stimulatory molecule is CD40 or B7.1.

36. A vaccine composition comprising cells or membranous material having engrafted thereto molecules capable of modifying an immunological response to a subject to which the vaccine is administered, said vaccine further comprising one or more pharmaceutical carriers and/or diluents.

37. A vaccine according to Claim 36 wherein the molecules engrafted to the cells or membranous material are co-stimulatory molecules.

38. A vaccine according to Claim 36 or 37 prepared by the steps of:-

(i) incubating the liposomes, cells or membranous material with a chelator lipid such as NTA-DTDA to allow the lipid to incorporate in the cells or membranes;

(ii) washing off any unincorporated lipid by centrifugation or filtration and resuspension of the liposomes, cells or membranous structures in the appropriate solution or buffer;

(iii) incubating the liposomes, cells or membranous structures with incorporated chelator lipid with said molecules to be engrafted; and

(iv) washing off unincorporated molecular material.

35. A method according to Claim 31 wherein the co-stimulatory molecule is CD40 or B7.1.

36. A vaccine composition comprising cells or membranous material having engrafted thereto molecules capable of modifying an immunological response to a subject to which the vaccine is administered, said vaccine further comprising one or more pharmaceutical carriers and/or diluents.

37. A vaccine according to Claim 36 wherein the molecules engrafted to the cells or membranous material are co-stimulatory molecules.

38. A vaccine according to Claim 36 or 37 prepared by the steps of:-

(i) incubating the liposomes, cells or membranous material with a chelator lipid such as NTA-DTDA to allow the lipid to incorporate in the cells or membranes;

(ii) washing off any unincorporated lipid by centrifugation or filtration and resuspension of the liposomes, cells or membranous structures in the appropriate solution or buffer;

(iii) incubating the liposomes, cells or membranous structures with incorporated chelator lipid with said molecules to be engrafted; and

(iv) washing off unincorporated molecular material.

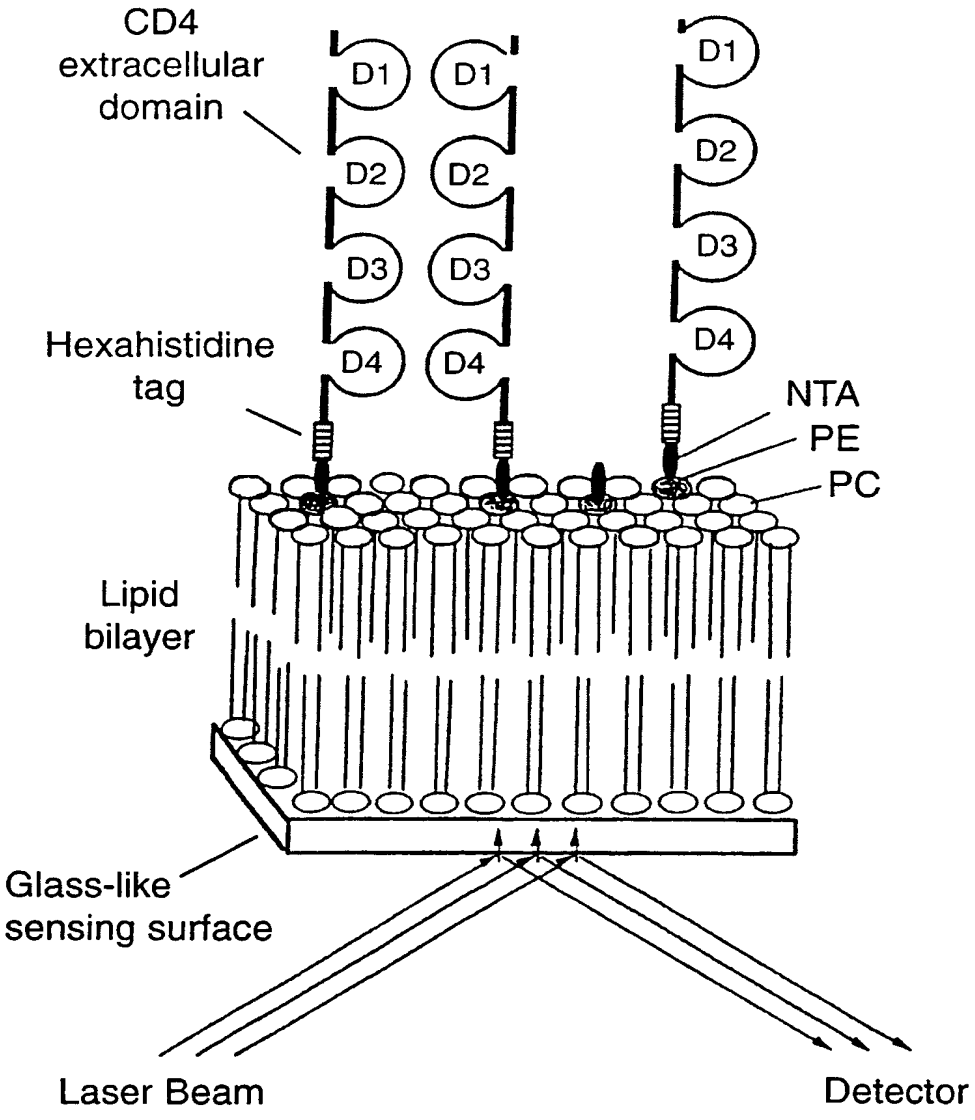

CD4
extracellular
domain
D1
D2
D3
D4
Hexahistidine
tag
NTA
PE
PC
Lipid
bilayer
Glass-like
sensing surface
Laser Beam
Detector

Figure 1

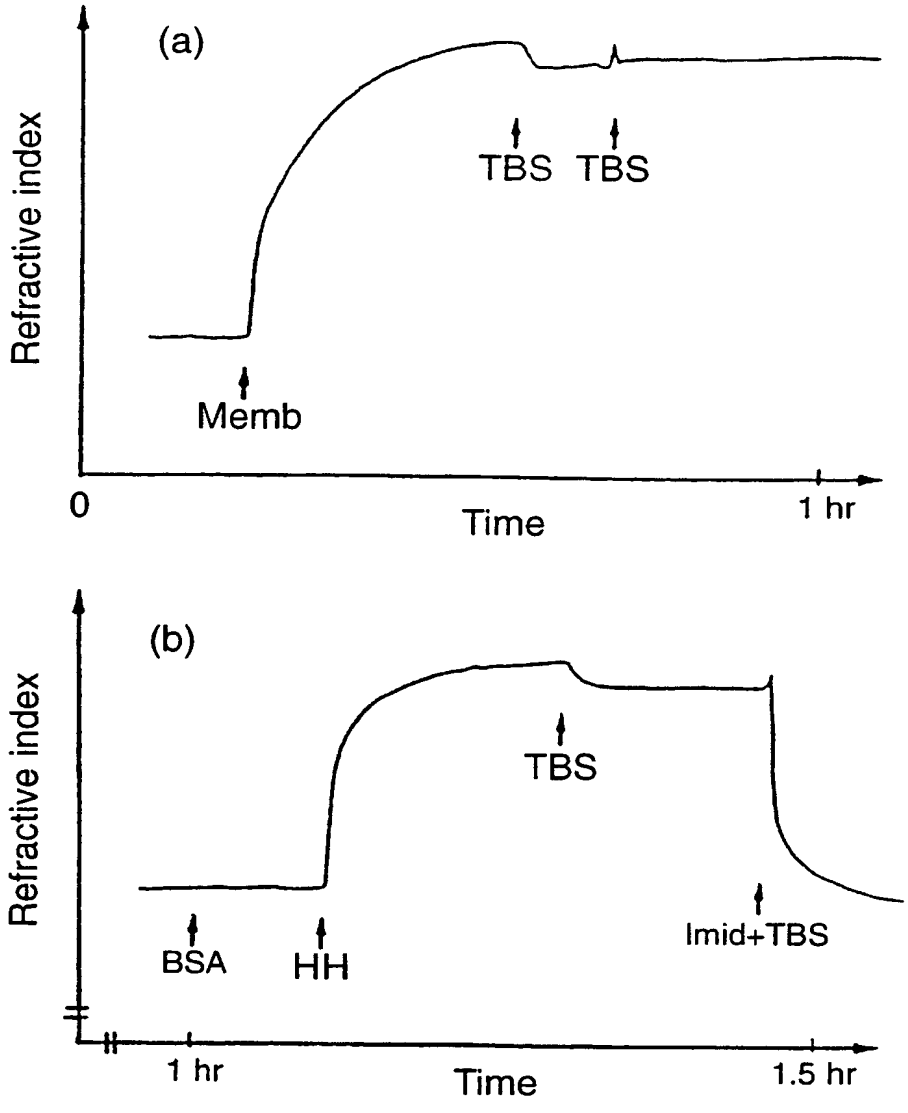
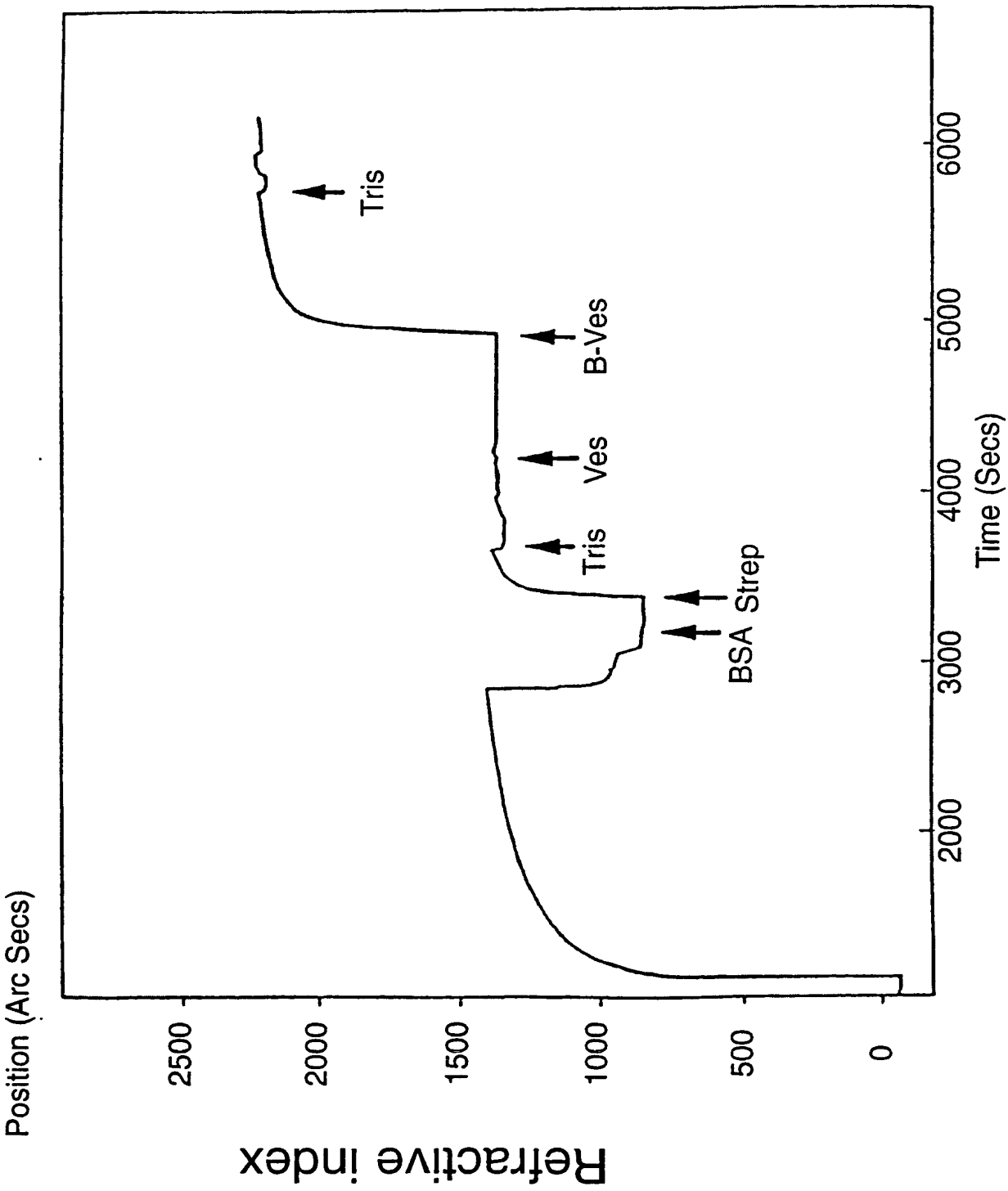

(a)
Refractive index
TBS TBS
Memb
0
Time
1 hr
(b)
Refractive index
TBS
BSA
HH
Imid+TBS
1 hr
Time
1.5 hr

Figure 2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Refractive index
2500
2000
1500
1000
500
0
2000
3000
4000
5000
6000
Time (Secs)
Tris
B-Ves
Ves
Tris
BSA Strep

Figure 3

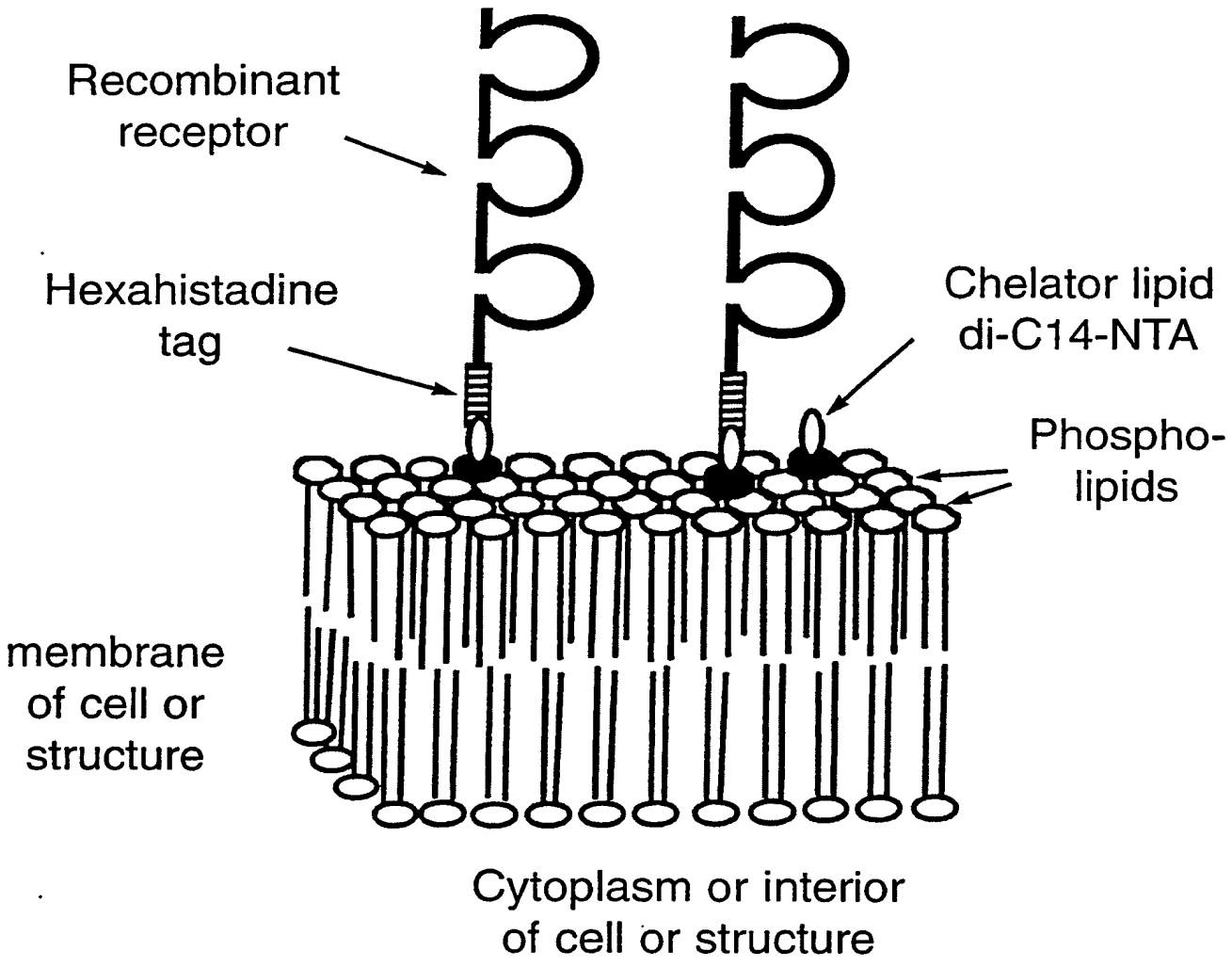
Recombinant receptor
Hexahistadine tag
Chelator lipid di-C14-NTA
Phospho-lipids
membrane of cell or structure
Cytoplasm or interior of cell or structure

Figure 4

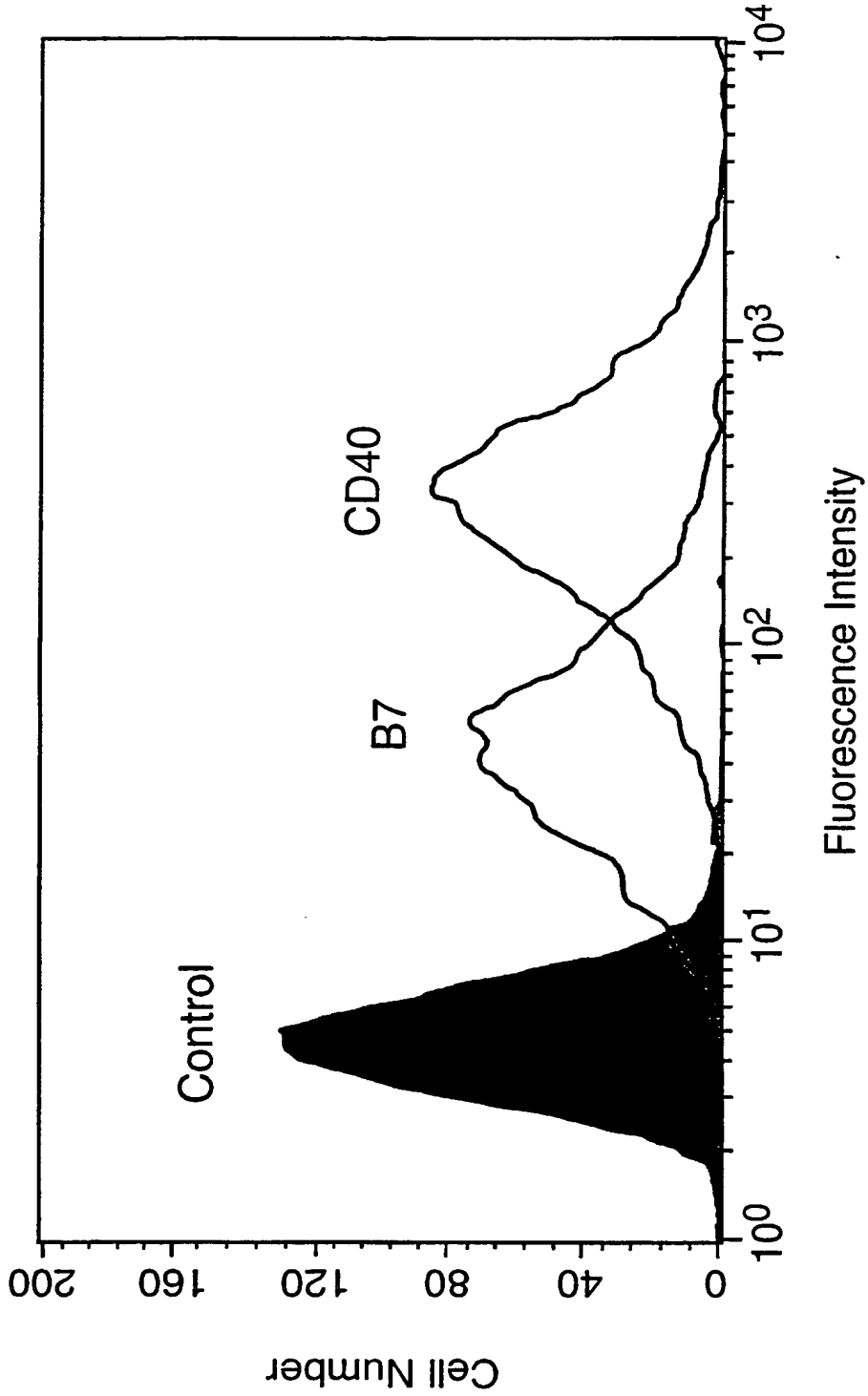
Cell Number
0
40
80
120
160
200
Fluorescence Intensity
10^0
10^1
10^2
10^3
10^4
Control
B7
CD40

Figure 5

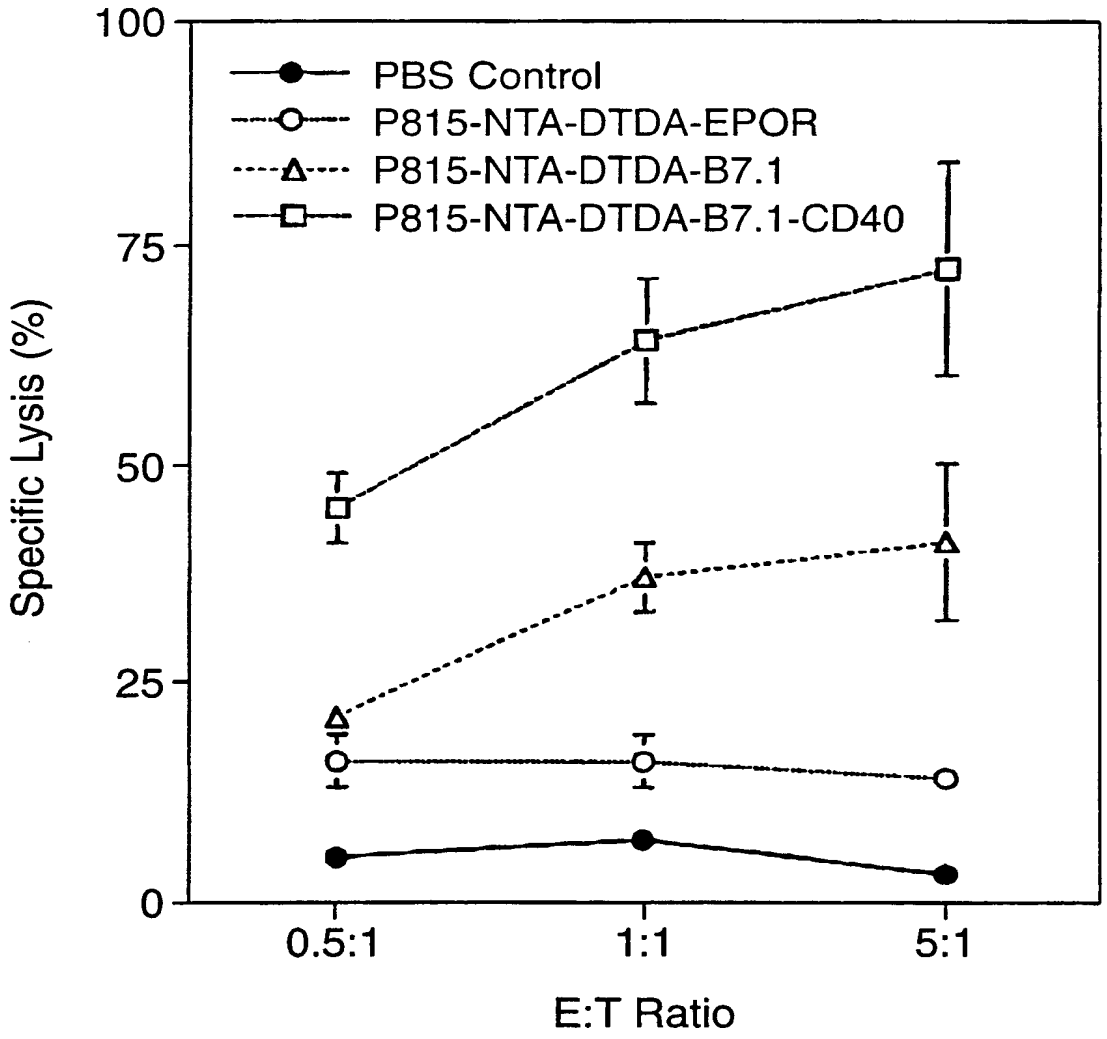
100
75
50
25
0
Specific Lysis (%)
PBS Control
P815-NTA-DTDA-EPOR
P815-NTA-DTDA-B7.1
P815-NTA-DTDA-B7.1-CD40
0.5:1
1:1
5:1
E:T Ratio

Figure 6

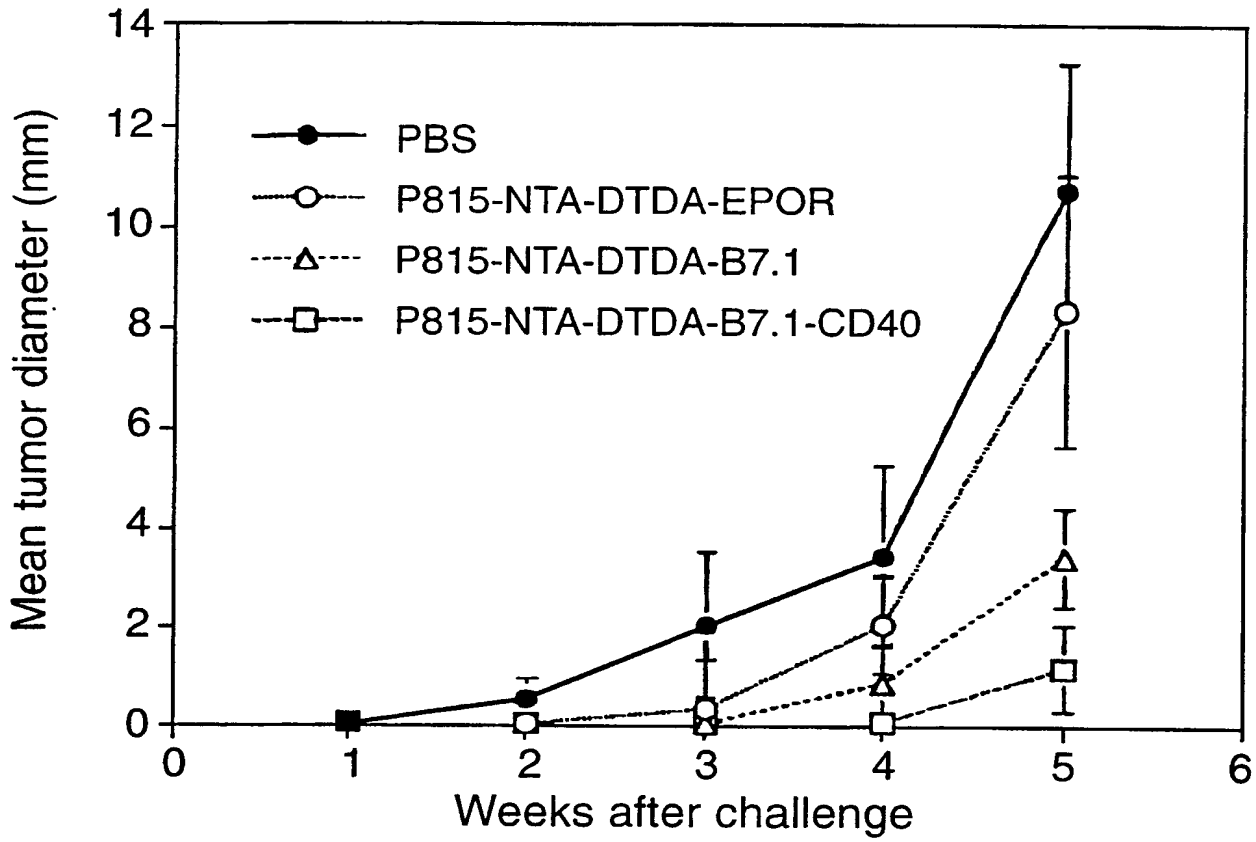
Mean tumor diameter (mm)
14
12
10
8
6
4
2
0
0
1
2
3
4
5
6
Weeks after challenge
PBS
P815-NTA-DTDA-EPOR
P815-NTA-DTDA-B7.1
P815-NTA-DTDA-B7.1-CD40

Figure 7a

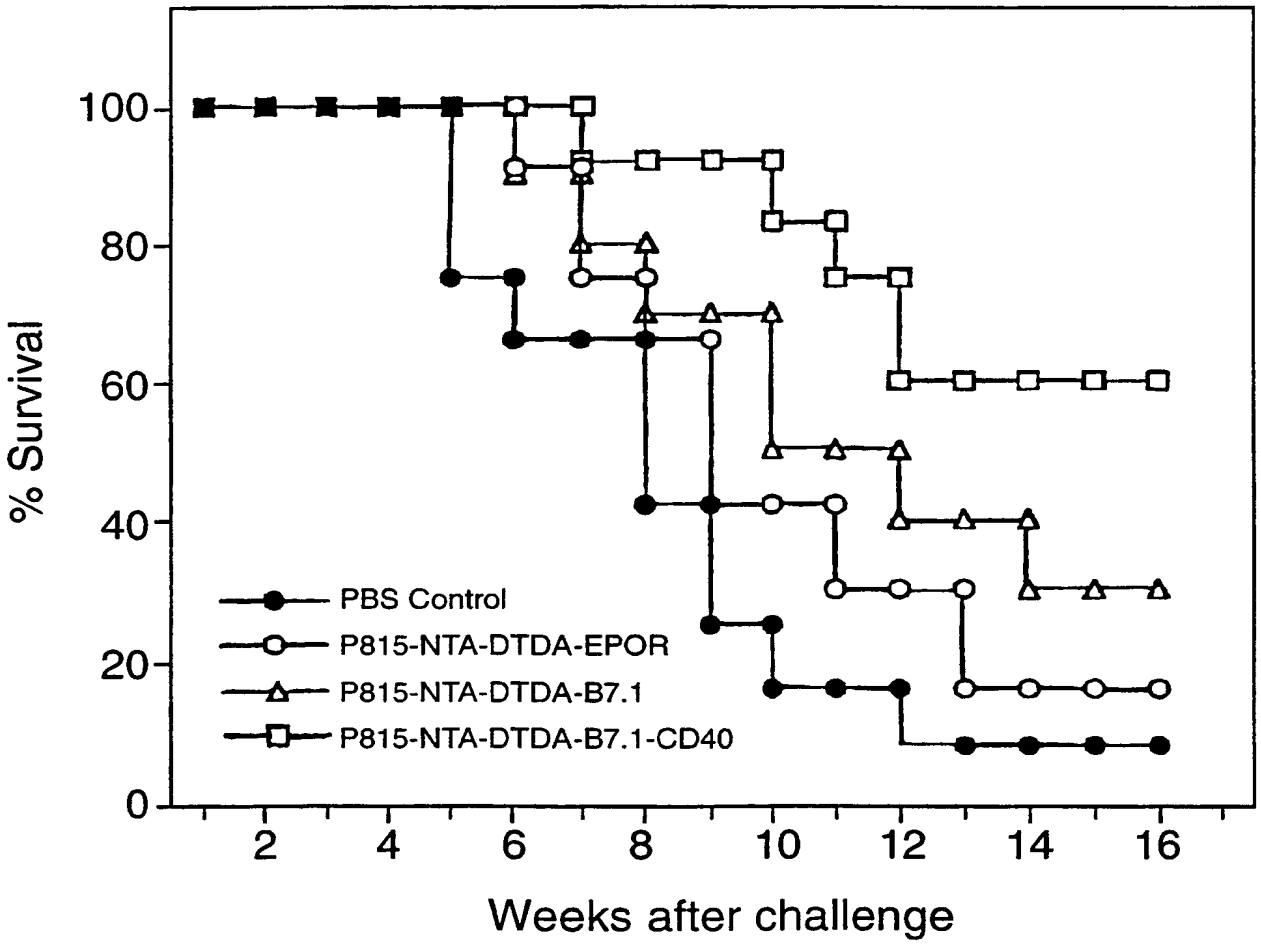
% Survival
100
80
60
40
20
0
2
4
6
8
10
12
14
16
Weeks after challenge
PBS Control
P815-NTA-DTDA-EPOR
P815-NTA-DTDA-B7.1
P815-NTA-DTDA-B7.1-CD40

Figure 7b

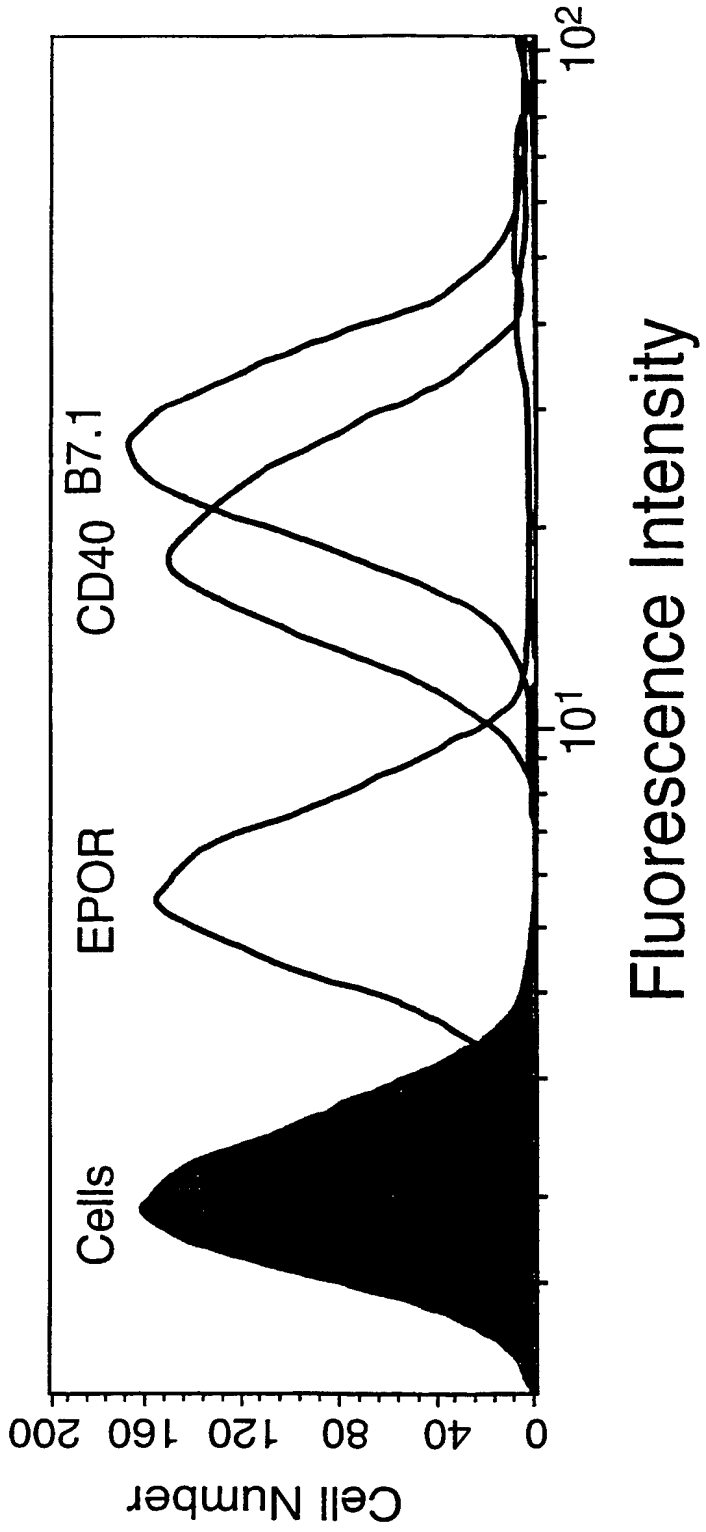
Cell Number
0
40
80
120
160
200
Cells
EPOR
CD40
B7.1
10^1
10^2
Fluorescence Intensity

Figure 8

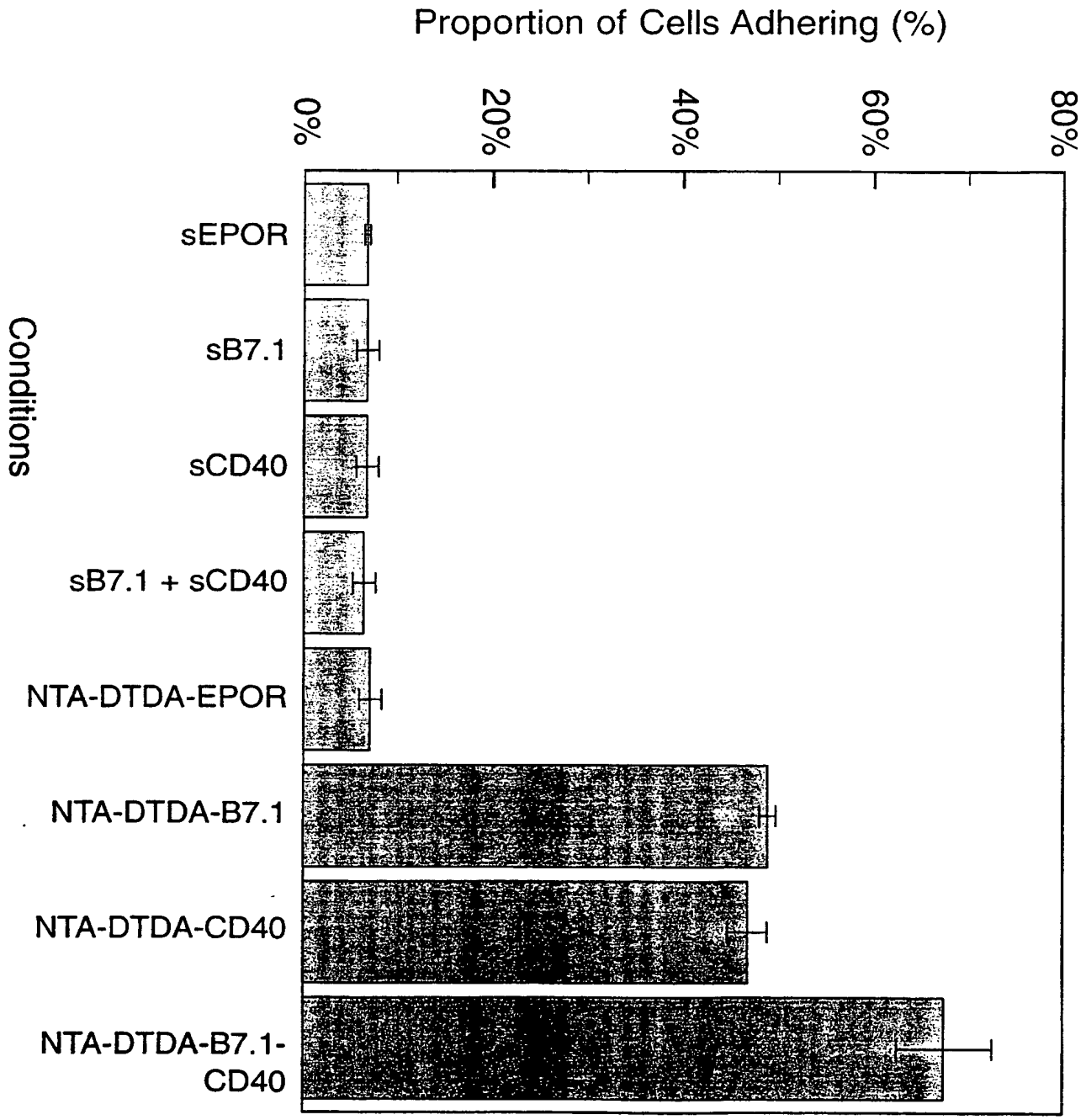
Proportion of Cells Adhering (%)
0%
20%
40%
60%
80%
Conditions
sEPOR
sB7.1
sCD40
sB7.1 + sCD40
NTA-DTDA-EPOR
NTA-DTDA-B7.1
NTA-DTDA-CD40
NTA-DTDA-B7.1-CD40

Figure 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00397

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 39/295, A61K 39/39, C07K 17/06, C07K 17/14, C12N 11/06, C12N 11/14, G01N 33/483, G01N 33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC A61K, C07K, C12N, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN WPIL JAPIO CAPLUS MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Am. Chem. Soc., vol.120, no.12, 2753-63; Dorn, I.T. et al. (1998) Molecular Recognition of Histidine-Tagged Molecules by Metal-Chelating Lipids Monitored by Fluorescence Energy Transfer and Correlation Spectroscopy. See in particular 'Molecular Docking at Vesicles'.	1-38
X,Y	Langmuir, vol. 14, no.17, 4836-42; Dorn, I.T. et al. (1998) Diacetylene Chelator Lipids as Support for Immobilization and Imaging of Proteins by Atomic Force Microscopy. See in particular 'Results and Discussion'.	1-38
X,Y	Langmuir, vol.11, no.10, 4048-55; Ng, K. et al. (1995) Engineering Protein-Lipid Interactions: Targeting of Histidine-Tagged Proteins to Metal-Chelating Lipid Monolayers. See in particular 'Discussion'.	1-38

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2000	26 JUN 2000
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**DAVID HENNESSY** Telephone No : (02) 6283 2255

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00397

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X,Y	Journal of Structural Biology, vol. 127, 44-52; Levy, D. et al. (1999) Two-Dimensional Crystallization on Lipid Layer: A Successful Approach for Membrane Proteins. See the abstract in particular.	1-38
X,Y	Biosensors & Bioelectronics, vol. 10, 805-12; Gritsch, S. et al. (1995) Engineered fusion molecules at chelator lipid interfaces imaged by reflection interference contrast microscopy (RICM). See the whole article.	1-38
X,Y	Biochemistry, vol. 35, no.4, 1100-5; Dietrich, C. et al. (1996) Functional Immobilization of a DNA-Binding Protein at a Membrane Interface via Histidine Tag and Synthetic Chelator Lipids. See the 'Discussion' in particular.	1-38
X,Y	Biophysical Journal, vol. 74, 1522-32; Bischler, N. et al. (1998) Specific Interaction and Two-Dimensional Crystallization of Histidine Tagged Yeast RNA Polymerase I on Nickel-Chelating Lipids. See the entire article.	1-38
X,Y	Proc.Natl.Acad.Sci., vol.92, 9014-18; Dietrich, C. et al. (1995) Molecular organisation of histidine-tagged biomolecules at self-assembled lipid interfaces using a novel class of chelator lipids. See the 'Discussion' in particular.	1-38
X,Y	J. Am. Chem. Soc., vol. 116, no.19, 8485-91; Schmitt, L. et al. (1994) Synthesis and Characterization of Chelator-Lipids for Reversible Immobilization of Engineered Proteins at Self-Assembled Lipid Interfaces. See the entire article.	1-38
Y	Medline Abstract PMID 10347753, UI 99277101, Braz. J. Med. Biol. Res., vol.32, no.2, 181-9; Frezard, F. (1999) Liposomes: from biophysics to the design of peptide vaccines, February 1999.	30-38

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00397

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

I. Claims 1-29; NTA - Ni polyhistidine tagged lipid systems;
II. Claims 30-38; liposome bound vaccines, adjuvants and methods of treatment.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

AMENDED CLAIMS

[received by the International Bureau on 25 August 2000 (25.08.00);
original claims 1-14 replaced by;
new claims 1-14; amended claims 15-38 (8 pages)]

CLAIMS

1. A method of targeting a membranous structure such as a biological or synthetic membrane, cell or organelle, to a specific cell type or tissue *in vivo*, said method comprising incorporating into said membranous structure, a chelator lipid and/or amphiphilic molecule which facilitates anchoring targeting molecules such as receptors, to allow the membranous structures to be targeted and be used to modify a biological or physiological response.

2. A method according to Claim 1 wherein the amphiphilic molecule is modified by a covalent attachment of a metal chelating group, with metal chelating groups orientated toward the outside surface of said membranous structure.

3. A method according to Claim 2 comprising the steps of:

(i) incorporating the chelator lipid into the membranous structure by mixing and/or co-incubation, or by production of the membrane from a composite mixture of lipids comprising a chelator lipid and one or more other lipids or phospholipids; and

(ii) interacting a targeting molecule with said membranous structure for a sufficient time and under suitable conditions to attach to said membranous structure *via* the outwardly facing metal chelating residues of said membranous structure, such that the receptor domains or targetable molecules are capable of lateral movement, dimerization/oligomerization and/or interaction with a ligand.

4. A method according to Claim 3 wherein the targeting molecule is a receptor domain and/or other targetable molecule engineered to possess a metal binding polypeptide tag.

5. A method according to Claim 4 wherein the receptor domain or targetable molecule is interacted with a membranous structure for a time and under conditions sufficient for the polypeptide tag to bind to the chelator lipid incorporated into the membranous structure.

6. A method according to Claim 1 or 2 or 3 or 4 or 5 wherein the membranous structure is a suspension of micelles or liposomes formed from the amphiphilic molecules by sonication, or extrusion/filtration techniques.

7. A method according to Claim 2 wherein the metal chelating group is nitrilotriacetic (NTA).

8. A method according to Claim 1 wherein a proportion of the amphiphilic molecules have been modified by a covalent attachment of a metal chelating group.

9. A method according to Claims 1 or 8 wherein the amphiphilic molecules are surfactant molecules having a hydrophilic head portion and one or more hydrophobic tails.

10. A method according to any one of the preceding claims wherein the polypeptide tag comprises a sequence of amino acid residues.

11. A method according to claim 10 wherein the amino acid residues are histidine residues.

12. A method to claim 10 or 11 wherein the polypeptide tag comprises at least five amino acid residues.

13. A method according to claim 12 wherein the polypeptide tag comprises at least six amino acid residues.

14. A method according to claim 13 wherein the polypeptide tag comprises hexa-histidine.

15. A method of modifying biological or synthetic membranes for: (i) vaccine development; (ii) for modification of biological response(s); and/or (iii) for the targeting of drugs or agents to specific tissue or cell types within the body to achieve a therapeutic effect, said method comprising:-

(i) preparing a suspension of liposomes with chelator lipid incorporated and with or without an encapsulated drug or agent;

(ii) incubating the liposomes with a recombinant protein or target molecule bearing an appropriate metal affinity tag; and

(iii) if necessary, removing excess protein or molecule by washing, filtering or other washing means and suspending them in an appropriate solution.

16. A method according to Claim 15 wherein the molecules engrafted, anchored or encapsulated within the liposome are therapeutic molecules, pharmaceutical compounds, DNA and/or RNA.

17. A method according to Claim 16 wherein the molecules engrafted or anchored onto the liposome surface is VEGF or its homologue.

18. A method according to Claim 17 wherein the liposome is encapsulated with a cytotoxic drug or agent together with the engrafted VEGF or its homologue to block the growth of new blood vessels for the growth of tumours.

19. A method according to Claim 15 wherein the liposome comprises an immunogenic agent and together with an agent which targets the liposome to different cell types in the body including immune cells and tumor cells to alter immunogenicity or immunological responses.

20. A method of anchoring a recombinant molecule directly onto biological membranes, said method comprising:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or biological membranous structures with a suspension of the chelator lipid to allow the chelator lipid to incorporate into the structures;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the membranous structures with a solution of recombinant protein or target molecule possessing an appropriate metal affinity tag; and

(v) washing away excess or unbound soluble protein, and suspending the structures in a solution suitable for administration in vivo.

21. A method according to Claim 20 wherein the recombinant molecule is a co-stimulatory molecule.

22. A method according to Claim 20 or 21 wherein the biological membrane is from a tumor cell.

23. A method according to Claim 20 or 21 or 22 for use in enhancing or modifying immunity to tumors or other disease conditions.

24. A method according to Claim 20 wherein the recombinant molecule is a receptor or ligand.

25. A method according to Claim 24 wherein the recombinant molecule is a ligand for a receptor on specific cell types within the body or on cells such as tumor cells that arise as a consequence of disease.

26. A method for altering the immunogenicity of a target cell or membranous component thereof, said method comprising anchoring a molecule to the membrane of said target cell by:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or membranous structures with a suspension of the chelator lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the membranous structures with a solution of said molecule to be anchored; and

(v) washing away excess or unbound soluble molecule, and suspending the structures in a solution suitable for administration *in vivo*.

27. A method according to Claim 26 wherein the target cell is a tumor cell.

28. A method according to Claim 26 or 27 wherein the molecule is a ligand, receptor, recombinant protein, polysaccharide, glycoprotein or antigen.

29. A method of targeting a liposome or a biological membranous structure to a particular cell or tissue, said method comprising anchoring or engrafting a molecule having a binding partner on the particular cell or tissue to be targeted by:-

(i) preparing a suspension of chelator lipid or liposomes containing the chelator lipid;

(ii) incubating a suspension of cells or biological membranous structures with a suspension of the chelator lipid;

(iii) washing away excess or unincorporated lipid;

(iv) incubating the liposomes or membranous structures with a solution of molecules to be anchored; and

(v) washing away excess or unbound soluble molecule, and suspending the structures in a solution suitable for administration *in vivo*.

30. A method of treatment, said method comprising administering to a subject an effective amount of a liposome preparation or membranous material comprising an active material and optionally an anchored or engrafted molecule having a binding partner or target tissue.

31. A method according to Claim 30 wherein the active material is a recombinant polypeptide, co-stimulatory molecule, therapeutic drug or nucleic acid molecule, either engrafted onto the surface or encapsulated within the liposome or membranous material.

32. A method according to Claim 30 or 31 wherein the anchored or engrafted molecule is a receptor, ligand, glycoprotein, polysaccharide or recombinant polypeptide.

33. A method according to Claim 32 wherein the anchored molecule is VEGF.

34. A method according to any one of Claims 26 to 33 when used to enhance immunity to a specific tumor or disease.

COPY

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
2269323		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/AU00/00397	28 April 2000	28 April 1999

Applicant

THE AUSTRALIAN NATIONAL UNIVERSITY et al

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☒ **Unity of invention is lacking** (See Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure

☐ because this figure better characterizes the invention

☒ None of the figures

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00397

Box I Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

I. Claims 1-29; NTA - Ni polyhistidine tagged lipid systems;
II. Claims 30-38; liposome bound vaccines, adjuvants and methods of treatment.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00397

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 39/295, A61K 39/39, C07K 17/06, C07K 17/14, C12N 11/06, C12N 11/14, G01N 33/483, G01N 33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC A61K, C07K, C12N, G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN WPIL JAPIO CAPLUS MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Am. Chem. Soc., vol.120, no.12, 2753-63; Dorn, I.T. et al. (1998) Molecular Recognition of Histidine-Tagged Molecules by Metal-Chelating Lipids Monitored by Fluorescence Energy Transfer and Correlation Spectroscopy. See in particular 'Molecular Docking at Vesicles'.	1-38
X,Y	Langmuir, vol. 14, no.17, 4836-42; Dorn, I.T. et al. (1998) Diacetylene Chelator Lipids as Support for Immobilization and Imaging of Proteins by Atomic Force Microscopy. See in particular 'Results and Discussion'.	1-38
X,Y	Langmuir, vol.11, no.10, 4048-55; Ng, K. et al. (1995) Engineering Protein-Lipid Interactions: Targeting of Histidine-Tagged Proteins to Metal-Chelating Lipid Monolayers. See in particular 'Discussion'.	1-38

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2000	26 JUN 2000
Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	**DAVID HENNESSY** Telephone No : (02) 6283 2255

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/00397

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X,Y	Journal of Structural Biology, vol. 127, 44-52; Levy, D. et al. (1999) Two-Dimensional Crystallization on Lipid Layer: A Successful Approach for Membrane Proteins. See the abstract in particular.	1-38
X,Y	Biosensors & Bioelectronics, vol. 10, 805-12; Gritsch, S. et al. (1995) Engineered fusion molecules at chelator lipid interfaces imaged by reflection interference contrast microscopy (RICM). See the whole article.	1-38
X,Y	Biochemistry, vol. 35, no.4, 1100-5; Dietrich, C. et al. (1996) Functional Immobilization of a DNA-Binding Protein at a Membrane Interface via Histidine Tag and Synthetic Chelator Lipids. See the 'Discussion' in particular.	1-38
X,Y	Biophysical Journal, vol. 74, 1522-32; Bischler, N. et al. (1998) Specific Interaction and Two-Dimensional Crystallization of Histidine Tagged Yeast RNA Polymerase I on Nickel-Chelating Lipids. See the entire article.	1-38
X,Y	Proc.Natl.Acad.Sci., vol.92, 9014-18; Dietrich, C. et al. (1995) Molecular organisation of histidine-tagged biomolecules at self-assembled lipid interfaces using a novel class of chelator lipids. See the 'Discussion' in particular.	1-38
X,Y	J. Am. Chem. Soc., vol. 116, no.19, 8485-91; Schmitt, L. et al. (1994) Synthesis and Characterization of Chelator-Lipids for Reversible Immobilization of Engineered Proteins at Self-Assembled Lipid Interfaces. See the entire article.	1-38
Y	Medline Abstract PMID 10347753, UI 99277101, Braz. J. Med. Biol. Res., vol.32, no.2, 181-9; Frezard, F. (1999) Liposomes: from biophysics to the design of peptide vaccines, February 1999.	30-38

14

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 2269323	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416).
International Application No. **PCT/AU00/00397**	International Filing Date *(day/month/year)* 28 April 2000	Priority Date *(day/month/year)* 28 April 1999

International Patent Classification (IPC) or national classification and IPC

Int. Cl. [7] A61K 39/295, 39/39, C07K 17/06, 17/14, C12N 11/06, 11/14, G01N 33/483, 33/68

Applicant

THE AUSTRALIAN NATIONAL UNIVERSITY et al

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

[X] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 59 sheet(s).

3. This report contains indications relating to the following items:

I	[X]	Basis of the report
II	[ ]	Priority
III	[ ]	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	[ ]	Lack of unity of invention
V	[X]	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
VI	[ ]	Certain documents cited
VII	[ ]	Certain defects in the international application
VIII	[ ]	Certain observations on the international application

Date of submission of the demand	Date of completion of the report
10 October 2000	26 July 2001
Name and mailing address of the IPEA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized Officer **DAVID HENNESSY** Telephone No. (02) 6283 2255

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
[redacted]T/AU00/00397

I. Basis of the report

1. With regard to the **elements** of the international application:*

- [ ] the international application as originally filed.
- [X] the description, pages , as originally filed,
  pages , filed with the demand,
  pages **1-44,** received on **9 March 2001** with the letter of **6 March 2001**
- [X] the claims, pages , as originally filed,
  pages , as amended (together with any statement) under Article 19,
  pages , filed with the demand,
  pages **45-52,** received on **9 March 2001** with the letter of **6 March 2001**
- [X] the drawings, pages , as originally filed,
  pages , filed with the demand,
  pages **1/7-7/7,** received on **9 March 2001** with the letter of **6 March 2001**
- [ ] the sequence listing part of the description:
  pages , as originally filed
  pages , filed with the demand
  pages , received on with the letter of

2. With regard to the **language,** all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.
These elements were available or furnished to this Authority in the following language which is:

- [ ] the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).
- [ ] the language of publication of the international application (under Rule 48.3(b)).
- [ ] the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- [ ] contained in the international application in written form.
- [ ] filed together with the international application in computer readable form.
- [ ] furnished subsequently to this Authority in written form.
- [ ] furnished subsequently to this Authority in computer readable form.
- [ ] The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- [ ] The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

4. [ ] The amendments have resulted in the cancellation of:
   - [ ] the description, pages
   - [ ] the claims, Nos.
   - [ ] the drawings, sheets/fig.

5. [ ] This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).**

* *Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).*

** *Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report*

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	19-20, 34 and 36	**YES**
	Claims	**1-18, 21-33, 35, 37-39**	**NO**
Inventive step (IS)	Claims		**YES**
	Claims	**1-39**	**NO**
Industrial applicability (IA)	Claims	**1-39**	**YES**
	Claims		**NO**

2. Citations and explanations (Rule 70.7)

Citations from the International Search Report:

D1 = J.Am.Chem.Soc, vol. 120, no.12, 2753-2763, Dorn et al. (1998);

D2 = Langmuir, vol.14, no.17, 4836-4842, Dorn et al. (1998);

D3 = Langmuir, vol.11, no. 10, 4048-4055, Ng et al. (1995);

D4 = Journal of Structural Biology, vol.127, 44-52, Levy et al. (1999);

D5 = Biosensors & Bioelectronics, vol.10, 805-812, Gritsch et al. (1995);

D6 = Biochemistry, vol.35, no.4, 1100-1105, Dietrich et al. (1996);

D7 = Biophysical Journal, vol.74, 1522-1532, Bischler et al. (1998);

D8 = Proc.Natl.Acad.Sci, vol.92, 9014-9018, Dietrich et al. (1995);

D9 = J.Am.Chem.Soc., vol.116, no.19, 8485-8491, Schmitt et al. (1994);

D10 = Medline Abstract PMID 10347753, UI 99277101, Frezard et al. (1999).

New Citations:

D11 = Proc.Natl.Acad.Sci., vol.94, 8795-8800, Spragg, D.D. et al. (1997) Immunotargetting of liposomes to activated vascular endothelial cells: A strategy for site selective delivery in the cardiovascular system;

D12 = Biochemistry, vol.33, 11664-11670, Laukkanen, M. et al. (1994) Functional Immunoliposomes Harboring a Biosynthetically Lipid-Tagged Single-Chain Antibody.

Reasoning

In the letter dated 26 June 2001, the applicants admit 'it is not in dispute that some prior art documents describe the generation of MTA-terminated lipid bilayers. It is further not in dispute that liposomes have been proposed for use in vaccines but this is not the essence of the invention. The essence of the invention is targeting...Targeting of membranous structures has been enabled by the present inventors by the use of chelator lipids...This then allows the membranous structures to be targeted to particular tissues in order to modify a biological or physiological response.'

Continued in the Supplemental Box.

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of V.

Citations

D1 relates to the stability of NTA lipid chelator-his tagged protein complexes measured by fluorescence with vesicles.

D2 is largely concerned with protein imaging using NTA lipid chelator-his tagged protein complexes, but also discloses that 'the polymerised chelator lipid layer is biocompatible' and 'this immobilisation technique preserves the functionality of the bound proteins'.

D3 discloses the study of the physical properties of NTA lipid chelator-his tagged protein complexes.

D4 is similar to D3, but is a 2D crystallography study.

D5 is similar to D3 and D4.

D6 discloses the NTA chelator lipid immobilisation of a DNA binding protein does not decrease its function, and states 'an imminent advantage lies in the high generality of this noval binding concept which allows the application to various proteins to study molecular and cellular recognition processes at membranes'.

D7 discloses the successful immobilisation of yeast RNA polymerase with NTA chelator lipid immobilisation.

D8 is similar to D2-D5.

D9 discloses the formation of NTA chelator lipid-histidine tagged protein putatively for 'biofunctionalisation of membranes, for liposome targetting with antibodies, for drug delivery systems, for immobilising receptors on lipid surfaces and in biosensor applications'.

D10 discloses that liposomes are used as drug delivery systems, including for the delivery of antigens.

D11 discloses that therapeutic targeted liposomes may be generated using antibodies conjugated to sterically stabilised liposomes.

D12 discloses that a lipid conjugated histidine tagged antibody is fully functional in an immunoliposome complex and may be used therapeutically.

Novelty (N) Claims 1-39

Claims 1-17, 21-33, 35, 37-39 lack novelty over any one of D6 or D9. Claims 31-33, 35, 37-39 lack novelty over any one of D10-D12. The citations disclose methods or vaccines within the scope of the claims. Claims 19-20, 34 and 36 are novel over the citations because the citations do not disclose the specific ligand molecules defined in the claims.

Inventive Step (IS) Claims 1-39

Claims 1-39 lack an inventive step over any one of D6 or D9-D12. The specific ligands defined in claims 19-20, 34 and 36 are not new. The application of the general method to these specific instances in not inventive (see the explanation of D6, D11 and D12 in particular).

Industrial Applicability (IA) Claims 1-39

Claims 1-39 have industrial applicability. Claims 31-36 are to methods of treatment, which may lack industrial applicability in some jurisdictions such as Europe.